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(57) Abstract

The present invention is directed to the use of particular gene combinations in genetic therapy. Delivery of multiple genes to a target cell at the same time augments the action of one or both genes. This is particularly effective in attacking diseased cells such as those making up hyperplastic or neoplastic tissues. Classes of genes that may be used in combination are tumor suppressors, cytokines and lymphokines, toxins, inducers of apoptosis, antisense oncogenes, single-chain antibodies, ribozymes, transcription factors and regulators, cell cycle regulators and enzymes.

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MULTIGENE VECTORS

BACKGROUND OF THE INVENTION

This application claims priority to and specifically incorporates by reference, the content of U.S. Provisional Application Serial No. 60/078,205 filed March 16, 1998. The entire text of each of the above-referenced disclosures is specifically incorporated by reference herein without disclaimer.

1. Field of the Invention

The present invention relates generally to the fields of gene transfer and gene therapy. More particularly, it concerns the development of viral vectors, particularly adenoviral vectors, that deliver specific combinations of genes to a target cell, *in vitro* or *in vivo*.

2. Description of Related Art

Gene replacement or augmentation is now considered a powerful tool in the therapeutic intervention of a variety of diseases and in particular, in cancer. The therapeutic treatment of diseases and disorders by gene therapy involves the transfer and stable or transient insertion of new genetic information into cells. The correction of a genetic defect by re-introduction of the normal allele of a gene encoding the desired function has demonstrated that this concept is clinically feasible (Rosenberg *et al.* (1990)). Indeed, preclinical and clinical studies covering a large range of genetic disorders currently are underway to solve basic issues dealing with gene transfer efficiency, regulation of gene expression, and potential risks of the use of viral vectors. The majority of clinical gene transfer trials that employ viral vectors perform *ex vivo* gene transfer into target cells which are then administered *in vivo*. Viral vectors also may be given *in vivo* but repeated administration may induce neutralizing antibody.

Of the different viral vectors attempted to mediate gene transfer, the retrovirus and adenovirus-based vector systems have been extensively investigated. Recently, adeno-associated virus (AAV) has emerged as a potential alternative to the more commonly

used retroviral and adenoviral vectors (Muzyczka, 1992; Carter, 1992; Flotte and Carter, 1995; Chatterjee *et al.*, 1995; Chatterjee and Wong, 1996). While studies with retroviral and adenoviral mediated gene transfer raise concerns over potential oncogenic properties of the former, and immunogenic problems associated with the latter, AAV has not been associated with any such pathological indications (Berns and Bohenzky, 1987; Berns and Giraud, 1996). However, the single-stranded nature of the AAV genome significantly impacts upon the transduction efficiency since the second-strand viral DNA synthesis is the rate-limiting step. Nonetheless, these vectors continue to be used extensively in gene therapy application.

It is important, therefore, in gene transfer therapies, to use as little of the viral vector as possible whilst effectively killing as many of the cells as quickly as possible. This can be achieved using combinations of gene therapy with other traditional therapies, as well as, with other gene therapies. Additional gene therapies require the use of separate vectors for each therapeutic construct, this presents a variety of problems including immunogenicity, oncogenicity and minimal transduction efficiency as described above. Further, the use of separate delivery vectors does not result in the consistent, reproducible expression of both genes in the same target cell.

It would be useful to develop a vector system that allows for the simultaneous delivery of two or more therapeutic genes to a target cell. Once such a vector system is elucidated, it will be possible to increase the efficiency and effectiveness of a gene based therapeutic intervention in, for example, hyperproliferative disorders.

SUMMARY OF THE INVENTION

Therefore, the present invention is directed to the use of particular gene combinations in genetic therapy. Delivery of multiple genes to a target cell at the same time augments the action of one or both genes. This is particularly effective in attacking diseased cells such as those making up hyperplastic or neoplastic tissues. Methods and

compositions for achieving such simultaneous delivery are described in detail herein below.

In a preferred embodiment, the present invention provides an expression construct comprising at least two different genes selected from the group consisting of a tumor suppressor and a cytokine, a tumor suppressor and an enzyme, a tumor suppressor and an antisense oncogene, a tumor suppressor and a toxin, a cytokine and an toxin, a cytokine and an antisense oncogene, an antisense oncogene, a toxin and an enzyme and a toxin, a tumor suppressor and an inducer of apoptosis, a cytokine and an inducer of apoptosis, an antisense oncogene and an inducer of apoptosis, an enzyme and an inducer of apoptosis, and a toxin and an inducer of apoptosis; and a first promoter active in eukaryotic cells positioned 5' to the different genes.

In other embodiments, the expression construct may further comprise an internal ribosome entry site (IRES), wherein the IRES is positioned 3' to the upstream gene and 5' to the downstream gene. In alternative embodiments, the expression construct may further comprises a second promoter, wherein the second promoter is positioned 3' to the upstream gene and 5' to the downstream gene. In a particularly preferred embodiment, the expression construct comprises tumor suppressor and a cytokine. In alternative preferred embodiment, the expression construct comprises a tumor suppressor and an enzyme. In still further preferred embodiments, the expression construct comprises a tumor suppressor and an antisense oncogene. In yet another alternative embodiment, the expression construct comprises a tumor suppressor and a toxin. In another embodiments, the expression construct comprises a cytokine and an toxin. In yet another embodiment, the expression construct comprises a cytokine and an antisense oncogene. In still another embodiment, the expression construct comprises an antisense oncogene and a toxin. Another alternative provides an expression construct comprising an enzyme and a toxin. Also preferred is an expression construct comprising a tumor suppressor and an inducer of apoptosis. In yet another preferred embodiment, there is provided an expression construct comprising a cytokine and an inducer of apoptosis. In yet another alternative,

the expression construct comprises an antisense oncogene and an inducer of apoptosis. Also contemplated is an expression construct comprising an enzyme and an inducer of apoptosis. In yet another embodiment, the expression construct comprises a toxin and an inducer of apoptosis.

In those embodiments employing a tumor suppressor as part of the expression construct, the tumor suppressor may be any tumor suppressor known to those of skill in the art. In particularly preferred embodiment, the tumor suppressor may be selected from the group consisting of p53, p16, p21, Rb, p15, BRCA1, BRCA2, zac1, p73, MMAC1, ATM, HIC-1, DPC-4, FHIT, NF2, APC, DCC, ING1, NOEY1, NOEY2, PML, OVCA1, MADR2, WT1, PTEN, 53BP2, IRF-1 and C-CAM.

In those embodiments employing a cytokine as part of the expression construct, the cytokine may be any cytokine known to those of skill in the art. In especially preferred embodiments, the cytokine may be selected from the group consisting of IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, GM-CSF, G-CSF, TNF, β-interferon and γ-interferon.

Likewise, those embodiments employing an enzyme as part of the expression construct may employ an enzyme selected from the group consisting of cytosine deaminase, adenosine deaminase, hypoxanthine-guanine phosphoribosyltransferase, galactose-1-phosphate uridyltransferase, phenylalanine hydroxylase, glucocerbrosidase, collagenase, sphingomyelinase, α-L-iduronidase, glucose-6-phosphate dehydrogenase, HSV thymidine kinase and human thymidine kinase.

Particular embodiments employ oncogenes as part of the expression construct, any oncogene known to those of skill in the art may be used herein. In especially preferred embodiments, the oncogene may be selected from the group consisting of *ras, myc, neu, raf, erb, src, fms, jun, trk, ret, hst, gsp, bcl-2* and *abl*. In specific embodiments, the expression construct may comprise a toxin, in particularly preferred embodiments, the

toxin may be selected from the group consisting of ricin A chain, diphtheria toxin, pertussis toxin, Pseudomonas toxin, *E. coli* enterotoxin, and cholera toxin. In those embodiment employing an inducer of apoptosis, particularly preferred inducers of apoptosis may be selected from the group consisting of Bax, Bak, Bcl-X_s, Bik, Bid, Bad, Harakiri, TRAIL, SARP-2, AdE1b and an ICE-CED3 protease. Particularly preferred examples of gene combinations are listed in Table 1 herein below. Of course these are only exemplary combinations and given the teachings of the present invention one of skill in the art will be able to produce multigene constructs of any conceivable combination, including but not limited to combinations of two, three, four five or more genes or nucleic acid constructs. By "nucleic acid constructs" the present invention refers to a nucleic acid the can encode a defined portion of or the whole of a particular gene.

In particularly preferred embodiments, the promoters used in the present invention may be selected from the group consisting of CMV IE, SV40 IE, RSV, human ubiquitin C, β-actin, tetracycline regulatable and ecdysone regulatable. These promoters independently may be used as the first promoter, or as the second or substituent promoter.

In particular aspects of the present invention the expression construct may further comprise a polyadenylation signal positioned 3' to the downstream gene. In particularly preferred embodiments, the expression construct may comprise a first polyadenylation signal positioned 3' to the upstream gene and 5' to the downstream gene and a second polyadenylation signal positioned 3' to the downstream gene. In particularly preferred embodiments, the polyadenylation signal may be from BGH, thymidine kinase or SV40. In more defined embodiments it is contemplated that the first polyadenylation signal is from BGH or SV40, and the second polyadenylation signal is from BGH when the first polyadenylation signal is from SV40, and the second polyadenylation signal is from SV40 when the first polyadenylation signal is from BGH.

In other aspects of the present invention, the expression construct may be a viral vector. In preferred embodiments, the viral vector may be selected from the group

consisting of retrovirus, adenovirus, vaccinia virus, herpesvirus and adeno-associated virus.

In those embodiments employing an adenoviral vector, it is contemplated that the adenovirus vector is replication deficient. In particularly preferred aspects, the adenovirus vector lacks at least a portion of the E1 region. In other especially preferred aspects the adenovirus lacks at least a portion of the E1B region. In alternative embodiments, the adenovirus lacks the entire E1 region.

Also contemplated by the present invention is an expression construct comprising a cytokine gene and an enzyme gene; and a first promoter active in eukaryotic cells positioned 5' to the genes, wherein either (i) the cytokine gene is not an IL-2 gene or (ii) the enzyme is not a herpesvirus thymidine kinase gene.

Further the present invention provides a method for the simultaneous expression of two polypeptides in a cell comprising providing an expression construct comprising at least two different genes selected from the group consisting of a tumor suppressor and a cytokine, a tumor suppressor and an enzyme, a tumor suppressor and an antisense oncogene, a tumor suppressor and a toxin, a cytokine and an toxin, a cytokine and an antisense oncogene, an antisense oncogene, a toxin and an enzyme and a toxin, a tumor suppressor and an inducer of apoptosis, a cytokine and an inducer of apoptosis, an antisense oncogene and an inducer of apoptosis, an enzyme and an inducer of apoptosis, and a toxin and an inducer of apoptosis; and a first promoter active in eukaryotic cells positioned 5' to the different genes; and transferring the expression construct into the cell, whereby expression of the gene is effected.

In particularly preferred embodiments, the expression construct is a viral vector and the transferring is achieved by viral infection. In other embodiments, the expression construct is formulated in a liposome and the transfer is achieved by cellular uptake of the liposome. In particular embodiments, the cell is a tumor cell and the cell is killed by

expression of the different genes. In more particular embodiments, the tumor cell is selected from the group consisting of a prostate cancer cell, a lung cancer cell, a brain cancer cell, a skin cancer cell, a liver cancer cell, a breast cancer cell, a lymphoid cancer cell, a stomach cancer cell, a testicular cancer cell, an ovarian cancer cell, a pancreatic cancer cell, a bone cancer cell, a bone marrow cancer cell, a head and neck cancer cell, a cervical cancer cell, a colon cancer cell, a rectal cancer cell, a blood cancer cell, an esophagus cancer cell, an eye cancer cell, a gall bladder cancer cell, a kidney cancer cells, an adrenal cancer cell and heart cancer cell.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1A and FIG. 1B - Multigene constructs. FIG. 1A: Single cassette, multiple promoter construct; FIG. 1B: Single, cassette, single promoter construct;

FIG. 2 - Cloning vector pIN147.

FIG. 3 - Cloning vector(s) pΔE1sp1A/B.

FIG. 4 - Multiple cassette construct pAB26.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

I. The Present Invention

Gene therapy now is becoming a viable alternative to various conventional therapies, especially in the area of cancer treatment. Limitations such as long term expression of transgenes and immuno-destruction of target cells through the expression of vector products, which have been said to limit the implementation of genetic therapies, are not concerns in cancer therapies, where destruction of cancer cells is desired.

It is important in gene transfer therapies, especially those involving treatment of cancer, to kill as many of the cells as quickly as possible. Thus, the use of "combination" therapies may be favored. Such combinations may include gene therapy and radiotherapy or chemotherapy. Roth *et al.* (1996) have demonstrated that a combination of DNA damaging agents and p53 gene therapy provides increased killing of tumor cells *in vivo*.

Yet another type of combination therapy involves the use of multi-gene therapy. In this situation, more than one therapeutic gene would be transferred into a target cell. The genes could be from the same functional group (*e.g.*, both tumor suppressors, both cytokines, *etc.*) or from different functional groups (*e.g.*, a tumor suppressor and a cytokine). By presenting particular combinations of therapeutic genes to a target cell, it may be possible to augment the overall effect of either or both genes on the physiology of the target cell.

The present invention seeks, therefore, to provide unique and advantageous combinations of genes for therapies, particularly where the destruction of a target cell is particularly desired. Such conditions include hyperproliferation, such as hyperplasia and benign and malignant neoplasias. The primary consideration in this endeavor is the combination of genes. The secondary consideration is how to achieve simultaneous

expression in a single cell of both therapeutic genes. The present inventors have chosen to approach this second issue by utilizing a single viral vector carrying both genes. Thus, infection of the cell by the vector ensures uptake and expression of both genes.

II. Viral Vectors

Although the methods and compositions described herein are directed to multigene adenoviral constructs, the methods and compositions described may be applicable to the construction of multigene constructs using other viral vectors including but not limited to retroviruses, herpes viruses, adeno-associated viruses, vaccinia viruses. The discussion below provides details regarding the characteristics of each of these viruses in relation to their application in therapeutic compositions.

A. Adenovirus

One of the preferred methods for *in vivo* delivery involves the use of an adenovirus expression vector. "Adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to express an antisense polynucleotide, a protein, a polynucleotide (*e.g.*, a ribozyme, or an mRNA) that has been cloned therein. In this context, expression does not require that the gene product be synthesized.

The expression vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization of adenovirus, a 36 kb, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kb (Grunhaus and Horwitz, 1992). In contrast to retroviruses, the adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. As used herein, the term "genotoxicity" refers to permanent inheritable host cell genetic alteration. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification of normal derivatives. Adenovirus can infect virtually all epithelial cells regardless of their cell cycle stage.

Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target cell range and high infectivity. Both ends of the viral genome contain 100-200 base pair inverted repeats (ITRs), which are *cis* elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression and host cell shut-off (Renan, 1990). The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP (located at 16.8 m.u.) is particularly efficient during the late phase of infection, and all the mRNA's issued from this promoter possess a 5'-tripartite leader (TPL) sequence which makes them preferred mRNA's for translation.

The E3 region encodes proteins that appear to be necessary for efficient lysis of Ad infected cells as well as preventing TNF-mediated cytolysis and CTL mediated lysis of infected cells. In general, the E4 region encodes is believed to encode seven proteins, some of which activate the E2 promoter. It has been shown to block host mRNA transport and enhance transport of viral RNA to cytoplasm. Further the E4 product is in part responsible for the decrease in early gene expression seen late in infection. E4 also inhibits E1A and E4 (but not E1B) expression during lytic growth. Some E4 proteins are necessary for efficient DNA replication however the mechanism for this involvement is unknown. E4 is also involved in post-transcriptional events in viral late gene expression; *i.e.*, alternative splicing of the tripartite leader in lytic growth. Nevertheless, E4 functions are not absolutely required for DNA replication but their lack will delay replication. Other functions include negative regulation of viral DNA synthesis, induction of sub-nuclear reorganization normally seen during adenovirus infection, and other functions

that are necessary for viral replication, late viral mRNA accumulation, and host cell transcriptional shut off.

In a current system, recombinant adenovirus is generated from homologous recombination between shuttle vector and provirus vector. Possible recombination between the proviral vector and Ad sequences in 293 cells, or in the case of pJM17 plasmid spontaneous deletion of the inserted pBR322 sequences, may generate full length wild-type Ad5 adenovirus. Therefore, it is critical to isolate a single clone of virus from an individual plaque and examine its genomic structure.

Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (Graham *et al.*, 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the E3 or both regions (Graham and Prevec, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury *et al.*, 1987), providing capacity for about 2 extra kb of DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kb, or about 15% of the total length of the vector. More than 80% of the adenovirus viral genome remains in the vector backbone and is the source of vector-borne cytotoxicity. Also, the replication deficiency of the E1-deleted virus is incomplete. For example, leakage of viral gene expression has been observed with the currently available vectors at high multiplicities of infection (MOI) (Mulligan, 1993; Shenk, 1978).

Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, *e.g.*,

Vero cells or other monkey embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Racher *et al.* (1995) disclosed improved methods for culturing 293 cells and propagating adenovirus. In one format, natural cell aggregates are grown by inoculating individual cells into 1 liter siliconized spinner flasks (Techne, Cambridge, UK) containing 100-200 ml of medium. Following stirring at 40 rpm, the cell viability is estimated with trypan blue. In another format, Fibra-Cel microcarriers (Bibby Sterlin, Stone, UK) (5 g/l) is employed as follows. A cell inoculum, resuspended in 5 ml of medium, is added to the carrier (50 ml) in a 250 ml Erlenmeyer flask and left stationary, with occasional agitation, for 1 to 4 h. The medium is then replaced with 50 ml of fresh medium and shaking is initiated. For virus production, cells are allowed to grow to about 80% confluence, after which time the medium is replaced (to 25% of the final volume) and adenovirus added at an MOI (pfu/mL) of 0.05. Cultures are left stationary overnight, following which the volume is increased to 100% and shaking commenced for another 72 h.

Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical, medical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

As stated above, the typical vector according to the present invention is replication defective and will not have an adenovirus E1 region. Thus, it will be most convenient to introduce the polynucleotide encoding the gene of interest at the position

from which the E1-coding sequences have been removed. However, the position of insertion of the construct within the adenovirus sequences is not critical to the invention. The polynucleotide encoding the gene of interest may also be inserted in lieu of the deleted E3 region in E3 replacement vectors as described by Karlsson *et al.* (1986), or in the E4 region where a helper cell line or helper virus complements the E4 defect.

Adenovirus is easy to grow and manipulate and exhibits broad host range *in vitro* and *in vivo*. This group of viruses can be obtained in high titers, *e.g.*, 10^9-10^{11} plaque-forming units per ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are episomal and, therefore, have low genotoxicity to host cells. No side effects have been reported in studies of vaccination with wild-type adenovirus (Couch *et al.*, 1963; Top *et al.*, 1971), demonstrating their safety and therapeutic potential as *in vivo* gene transfer vectors.

Adenovirus vectors have been used in eukaryotic gene expression investigations (Levrero *et al.*, 1991; Gomez-Foix *et al.*, 1992) and vaccine development (Grunhaus and Horwitz, 1992; Graham and Prevec, 1992). Recently, animal studies suggested that recombinant adenovirus could be used for gene therapy (Stratford-Perricaudet and Perricaudet, 1991; Stratford-Perricaudet *et al.*, 1990; Rich *et al.*, 1993). Studies in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld *et al.*, 1991; Rosenfeld *et al.*, 1992), muscle injection (Ragot *et al.*, 1993), peripheral intravenous injections (Herz and Gerard, 1993), intranasal inoculation (Ginsberg *et al.*, 1991), aerosol administration to lung (Bellon, 1996) intra-peritoneal administration (Song *et al.*, 1997), intra-pleural injection (Elshami *et al.*, 1996) administration to the bladder using intra-vesicular administration (Werthman, *et al.*, 1996), subcutaneous injection (Ogawa, 1989), ventricular injection into myocardium (heart, French *et al.*, 1994), liver perfusion (hepatic artery or portal vein, Shiraishi *et al.*, 1997) and stereotactic inoculation into the brain (Le Gal La Salle *et al.*, 1993).

B. Retrovirus

The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes, *gag*, *pol*, and *env* that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the *gag* gene contains a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5′ and 3′ ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding a gene of interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the *gag*, *pol*, and *env* genes but without the LTR and packaging components is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a cDNA, together with the retroviral LTR and packaging sequences is introduced into this cell line (by calcium phosphate precipitation for example), the packaging sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

A novel approach designed to allow specific targeting of retrovirus vectors was recently developed based on the chemical modification of a retrovirus by the chemical

addition of lactose residues to the viral envelope. This modification could permit the specific infection of hepatocytes *via* sialoglycoprotein receptors.

A different approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled *via* the biotin components by using streptavidin (Roux *et al.*, 1989). Using antibodies against major histocompatibility complex class I and class II antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus *in vitro* (Roux *et al.*, 1989).

There are certain limitations to the use of retrovirus vectors in all aspects of the present invention. For example, retrovirus vectors usually integrate into random sites in the cell genome. This can lead to insertional mutagenesis through the interruption of host genes or through the insertion of viral regulatory sequences that can interfere with the function of flanking genes (Varmus *et al.*, 1981). Another concern with the use of defective retrovirus vectors is the potential appearance of wild-type replication-competent virus in the packaging cells. This can result from recombination events in which the intact- sequence from the recombinant virus inserts upstream from the gag, pol, env sequence integrated in the host cell genome. However, new packaging cell lines are now available that should greatly decrease the likelihood of recombination (Markowitz *et al.*, 1988; Hersdorffer *et al.*, 1990).

C. Herpesvirus

Because herpes simplex virus (HSV) is neurotropic, it has generated considerable interest in treating nervous system disorders. Moreover, the ability of HSV to establish latent infections in non-dividing neuronal cells without integrating in to the host cell chromosome or otherwise altering the host cell's metabolism, along with the existence of a promoter that is active during latency makes HSV an attractive vector. And though

much attention has focused on the neurotropic applications of HSV, this vector also can be exploited for other tissues given its wide host range.

Another factor that makes HSV an attractive vector is the size and organization of the genome. Because HSV is large, incorporation of multiple genes or expression cassettes is less problematic than in other smaller viral systems. In addition, the availability of different viral control sequences with varying performance (temporal, strength, *etc.*) makes it possible to control expression to a greater extent than in other systems. It also is an advantage that the virus has relatively few spliced messages, further easing genetic manipulations.

HSV also is relatively easy to manipulate and can be grown to high titers. Thus, delivery is less of a problem, both in terms of volumes needed to attain sufficient MOI and in a lessened need for repeat dosings. For a review of HSV as a gene therapy vector, see Glorioso *et al.* (1995).

HSV, designated with subtypes 1 and 2, are enveloped viruses that are among the most common infectious agents encountered by humans, infecting millions of human subjects worldwide. The large, complex, double-stranded DNA genome encodes for dozens of different gene products, some of which derive from spliced transcripts. In addition to virion and envelope structural components, the virus encodes numerous other proteins including a protease, a ribonucleotides reductase, a DNA polymerase, a ssDNA binding protein, a helicase/primase, a DNA dependent ATPase, a dUTPase and others.

HSV genes form several groups whose expression is coordinately regulated and sequentially ordered in a cascade fashion (Honess and Roizman, 1974; Honess and Roizman 1975; Roizman and Sears, 1995). The expression of α genes, the first set of genes to be expressed after infection, is enhanced by the virion protein number 16, or α-transinducing factor (Post *et al.*, 1981; Batterson and Roizman, 1983; Campbell, *et al.*, 1983). The expression of β genes requires functional α gene products, most notably

ICP4, which is encoded by the α4 gene (DeLuca *et al.*, 1985). γ genes, a heterogeneous group of genes encoding largely virion structural proteins, require the onset of viral DNA synthesis for optimal expression (Holland *et al.*, 1980).

In line with the complexity of the genome, the life cycle of HSV is quite involved. In addition to the lytic cycle, which results in synthesis of virus particles and, eventually, cell death, the virus has the capability to enter a latent state in which the genome is maintained in neural ganglia until some as of yet undefined signal triggers a recurrence of the lytic cycle. Avirulent variants of HSV have been developed and are readily available for use in gene therapy contexts (U.S. Patent No. 5,672,344).

D. Adeno-Associated Virus

Recently, adeno-associated virus (AAV) has emerged as a potential alternative to the more commonly used retroviral and adenoviral vectors. While studies with retroviral and adenoviral mediated gene transfer raise concerns over potential oncogenic properties of the former, and immunogenic problems associated with the latter, AAV has not been associated with any such pathological indications.

In addition, AAV possesses several unique features that make it more desirable than the other vectors. Unlike retroviruses, AAV can infect non-dividing cells; wild-type AAV has been characterized by integration, in a site-specific manner, into chromosome 19 of human cells (Kotin and Berns, 1989; Kotin *et al.*, 1990; Kotin *et al.*, 1991; Samulski *et al.*, 1991); and AAV also possesses anti-oncogenic properties (Ostrove *et al.*, 1981; Berns and Giraud, 1996). Recombinant AAV genomes are constructed by molecularly cloning DNA sequences of interest between the AAV ITRs, eliminating the entire coding sequences of the wild-type AAV genome. The AAV vectors thus produced lack any of the coding sequences of wild-type AAV, yet retain the property of stable chromosomal integration and expression of the recombinant genes upon transduction both *in vitro* and *in vivo* (Berns, 1990; Berns and Bohensky, 1987; Bertran *et al.*, 1996; Kearns *et al.*, 1996; Ponnazhagan *et al.*, 1997a). Until recently, AAV was believed to

infect almost all cell types, and even cross species barriers. However, it now has been determined that AAV infection is receptor-mediated (Ponnazhagan *et al.*, 1996; Mizukami *et al.*, 1996).

AAV utilizes a linear, single-stranded DNA of about 4700 base pairs. Inverted terminal repeats flank the genome. Two genes are present within the genome, giving rise to a number of distinct gene products. The first, the *cap* gene, produces three different virion proteins (VP), designated VP-1, VP-2 and VP-3. The second, the *rep* gene, encodes four non-structural proteins (NS). One or more of these *rep* gene products is responsible for transactivating AAV transcription. The sequence of AAV is provided by Srivastava *et al.* (1983), and in U.S. Patent 5,252,479 (entire text of which is specifically incorporated herein by reference).

The three promoters in AAV are designated by their location, in map units, in the genome. These are, from left to right, p5, p19 and p40. Transcription gives rise to six transcripts, two initiated at each of three promoters, with one of each pair being spliced. The splice site, derived from map units 42-46, is the same for each transcript. The four non-structural proteins apparently are derived from the longer of the transcripts, and three virion proteins all arise from the smallest transcript.

AAV is not associated with any pathologic state in humans. Interestingly, for efficient replication, AAV requires "helping" functions from viruses such as herpes simplex virus I and II, cytomegalovirus, pseudorabies virus and, of course, adenovirus. The best characterized of the helpers is adenovirus, and many "early" functions for this virus have been shown to assist with AAV replication. Low level expression of AAV *rep* proteins is believed to hold AAV structural expression in check, and helper virus infection is thought to remove this block.

E. Vaccinia Virus

Vaccinia virus vectors have been used extensively because of the ease of their construction, relatively high levels of expression obtained, wide host range and large capacity for carrying DNA. Vaccinia contains a linear, double-stranded DNA genome of about 186 kb that exhibits a marked "A-T" preference. Inverted terminal repeats of about 10.5 kb flank the genome. The majority of essential genes appear to map within the central region, which is most highly conserved among poxviruses. Estimated open reading frames in vaccinia virus number from 150 to 200. Although both strands are coding, extensive overlap of reading frames is not common.

At least 25 kb can be inserted into the vaccinia virus genome (Smith and Moss, 1983). Prototypical vaccinia vectors contain transgenes inserted into the viral thymidine kinase gene *via* homologous recombination. Vectors are selected on the basis of a tk-phenotype. Inclusion of the untranslated leader sequence of encephalomyocarditis virus, the level of expression is higher than that of conventional vectors, with the transgenes accumulating at 10% or more of the infected cell's protein in 24 h (Elroy-Stein *et al.*, 1989).

III. Non-viral transfer

Although the present invention describes the use of adenoviral multigene constructs, the present invention may also employ non-viral gene transfer. This section provides a discussion of methods and compositions of non-viral gene transfer.

DNA constructs of the present invention are generally delivered to a cell, and in certain situations, the nucleic acid to be transferred may be transferred using non-viral methods.

Several non-viral methods for the transfer of expression constructs into cultured mammalian cells are contemplated by the present invention. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987;

Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), direct microinjection (Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979), cell sonication (Fechheimer *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990), and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988).

Once the construct has been delivered into the cell the nucleic acid encoding the therapeutic gene may be positioned and expressed at different sites. In certain embodiments, the nucleic acid encoding the therapeutic gene may be stably integrated into the genome of the cell. This integration may be in the cognate location and orientation *via* homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (gene augmentation). In yet further embodiments, the nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle. How the expression construct is delivered to a cell and where in the cell the nucleic acid remains is dependent on the type of expression construct employed.

In a particular embodiment of the invention, the expression construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). The addition of DNA to cationic liposomes causes a topological transition from liposomes to optically birefringent liquid-crystalline condensed globules (Radler *et al.*, 1997). These DNA-lipid complexes are potential non-viral vectors for use in gene therapy.

Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful. Using the β-lactamase gene, Wong *et al.* (1980) demonstrated the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa, and hepatoma cells. Nicolau *et al.* (1987) accomplished successful liposome-mediated gene transfer in rats after intravenous injection. Also included are various commercial approaches involving "lipofection" technology.

In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989). In other embodiments, the liposome may be complexed or employed in conjunction with nuclear nonhistone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In that such expression constructs have been successfully employed in transfer and expression of nucleic acid *in vitro* and *in vivo*, then they are applicable for the present invention.

Other vector delivery systems which can be employed to deliver a nucleic acid encoding a therapeutic gene into cells are receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific distribution of various receptors, the delivery can be highly specific (Wu and Wu, 1993).

Receptor-mediated gene targeting vehicles generally consist of two components: a cell receptor-specific ligand and a DNA-binding agent. Several ligands have been used for receptor-mediated gene transfer. The most extensively characterized ligands are asialoorosomucoid (ASOR) (Wu and Wu, 1987) and transferrin (Wagner *et al.*, 1990). Recently, a synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle (Ferkol *et al.*, 1993; Perales *et al.*, 1994) and

epidermal growth factor (EGF) has also been used to deliver genes to squamous carcinoma cells (Myers, EPO 0273085).

In other embodiments, the delivery vehicle may comprise a ligand and a liposome. For example, Nicolau *et al.* (1987) employed lactosyl-ceramide, a galactose-terminal asialganglioside, incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes. Thus, it is feasible that a nucleic acid encoding a therapeutic gene also may be specifically delivered into a cell type such as prostate, epithelial or tumor cells, by any number of receptor-ligand systems with or without liposomes. For example, the human prostate-specific antigen (Watt *et al.*, 1986) may be used as the receptor for mediated delivery of a nucleic acid in prostate tissue.

In another embodiment of the invention, the expression construct may simply consist of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. This is applicable particularly for transfer *in vitro*, however, it may be applied for *in vivo* use as well. Dubensky *et al.* (1984) successfully injected polyomavirus DNA in the form of $CaPO_4$ precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of $CaPO_4$ precipitated plasmids results in expression of the transfected genes. It is envisioned that DNA encoding a CAM may also be transferred in a similar manner *in vivo* and express CAM.

Another embodiment of the invention for transferring a naked DNA expression construct into cells may involve particle bombardment. This method depends on the ability to accelerate DNA coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive

force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

IV. Gene Combinations

A. Tumor Suppressors

p53 (designated **1** in Table 1) currently is recognized as a tumor suppressor gene. High levels of mutant p53 have been found in many cells transformed by chemical carcinogenesis, ultraviolet radiation, and several viruses. The p53 gene is a frequent target of mutational inactivation in a wide variety of human tumors and is already documented to be the most frequently-mutated gene in common human cancers. It is mutated in over 50% of human NSCLC (Hollstein *et al.*, 1991) and in a wide spectrum of other tumors.

The p53 gene encodes a 393-amino acid phosphoprotein that can form complexes with host proteins such as SV40 large-T antigen and adenoviral E1B. The protein is found in normal tissues and cells, but at concentrations which are minute by comparison with transformed cells or tumor tissue. Interestingly, wild-type p53 appears to be important in regulating cell growth and division. Overexpression of wild-type p53 has been shown in some cases to be anti-proliferative in human tumor cell lines. Thus, p53 can act as a negative regulator of cell growth (Weinberg, 1991) and may directly suppress uncontrolled cell growth or indirectly activate genes that suppress this growth. Thus, absence or inactivation of wild-type p53 may contribute to transformation. However, some studies indicate that the presence of mutant p53 may be necessary for full expression of the transforming potential of the gene.

Wild-type p53 is recognized as an important growth regulator in many cell types. Missense mutations are common for the p53 gene and are essential for the transforming ability of the oncogene. A single genetic change prompted by point mutations can create carcinogenic p53, in as much as mutations in p53 are known to abrogate the tumor suppressor capability of wild-type p53. Unlike other oncogenes, however, p53 point

mutations are known to occur in at least 30 distinct codons, often creating dominant alleles that produce shifts in cell phenotype without a reduction to homozygosity. Additionally, many of these dominant negative alleles appear to be tolerated in the organism and passed on in the germ line. Various mutant alleles appear to range from minimally dysfunctional to strongly penetrant, dominant negative alleles (Weinberg, 1991).

Casey and colleagues have reported that transfection of DNA encoding wild-type p53 into two human breast cancer cell lines restores growth suppression control in such cells (Casey *et al.*, 1991). A similar effect also has been demonstrated on transfection of wild-type, but not mutant, p53 into human lung cancer cell lines (Takahasi *et al.*, 1992). p53 appears dominant over the mutant gene and will select against proliferation when transfected into cells with the mutant gene. Normal expression of the transfected p53 does not affect the growth of normal or non-malignant cells with endogenous p53. Thus, such constructs might be taken up by normal cells without adverse effects. It is thus proposed that the treatment of p53-associated cancers with wild-type p53 will reduce the number of malignant cells or their growth rate.

The major transitions of the eukaryotic cell cycle are triggered by cyclin-dependent kinases, or CDK's. One CDK, cyclin-dependent kinase 4 (CDK4), regulates progression through the G_1. The activity of this enzyme may be to phosphorylate Rb at late G_1. The activity of CDK4 is controlled by an activating subunit, D-type cyclin, and by an inhibitory subunit $p16^{INK4}$. The $p16^{INK4}$ has been biochemically characterized as a protein that specifically binds to and inhibits CDK4, and thus may regulate Rb phosphorylation (Serrano *et al.*, 1993; Serrano *et al.*, 1995). Since the $p16^{INK4}$ protein is a CDK4 inhibitor (Serrano, 1993), deletion of this gene may increase the activity of CDK4, resulting in hyperphosphorylation of the Rb protein. p16 also is known to regulate the function of CDK6.

$p16^{INK4}$ belongs to a newly described class of CDK-inhibitory proteins that also includes $p15^{INK4B}$, $p21^{WAF1}$, and $p27^{KIP1}$. The $p16^{INK4}$ gene maps to 9p21, a chromosome region frequently deleted in many tumor types. Homozygous deletions and mutations of the $p16^{INK4}$ gene are frequent in human tumor cell lines. This evidence suggests that the $p16^{INK4}$ gene is a tumor suppressor gene. This interpretation has been challenged, however, by the observation that the frequency of the $p16^{INK4}$ gene alterations is much lower in primary uncultured tumors than in cultured cell lines (Caldas *et al.*, 1994; Cheng *et al.*, 1994; Hussussian *et al.*, 1994; Kamb *et al.*, 1994; Kamb *et al.*, 1994; Mori *et al.*, 1994; Okamoto *et al.*, 1994; Nobori *et al.*, 1995; Orlow *et al.*, 1994; Arap *et al.*, 1995). However, it was later shown that while the p16 gene was intact in many primary tumors, there were other mechanisms that prevented p16 protein expression in a large percentage of some tumor types. p16 promoter hypermethylation is one of these mechanisms (Merlo *et al.*, 1995; Herman, 1995; Gonzalez-Zulueta, 1995). Restoration of wild-type $p16^{INK4}$ function by transfection with a plasmid expression vector reduced colony formation by some human cancer cell lines (Okamoto, 1994; Arap, 1995). Delivery of p16 with adenovirus vectors inhibits proliferation of some human cancer lines and reduces the growth of human tumor xenografts.

C-CAM (designated 2 in Table 1) is expressed in virtually all epithelial cells (Odin and Obrink, 1987). C-CAM, with an apparent molecular weight of 105 kD, was originally isolated from the plasma membrane of the rat hepatocyte by its reaction with specific antibodies that neutralize cell aggregation (Obrink, 1991). Recent studies indicate that, structurally, C-CAM belongs to the immunoglobulin (Ig) superfamily and its sequence is highly homologous to carcinoembryonic antigen (CEA; designated 3 in Table 1) (Lin and Guidotti, 1989). Using a baculovirus expression system, Cheung *et al.* (1993) demonstrated that the first Ig domain of C-CAM is critical for cell adhesive activity.

Cell adhesion molecules, or CAM's are known to be involved in a complex network of molecular interactions that regulate organ development and cell differentiation

(Edelman, 1985). Recent data indicate that aberrant expression of CAM's maybe involved in the tumorigenesis of several neoplasms; for example, decreased expression of E-cadherin, which is predominantly expressed in epithelial cells, is associated with the progression of several kinds of neoplasms (Edelman and Crossin, 1991; Frixen *et al.*, 1991; Bussemakers *et al.*, 1992; Matsura *et al.*, 1992; Umbas *et al.*, 1992). Also, Giancotti and Ruoslahti (1990) demonstrated that increasing expression of $\alpha_5\beta_1$ integrin by gene transfer can reduce tumorigenicity of Chinese hamster ovary cells *in vivo*. C-CAM now has been shown to suppress tumor growth *in vitro* and *in vivo*.

Other tumor suppressors that may be employed according to the present invention include p21 (designated **4** in Table 1), p15 (designated **5** in Table 1), BRCA1 (designated **6** in Table 1), BRCA2 (designated **7** in Table 1), IRF-1 (designated **8** in Table 1), PTEN (MMAC1; designated **9** in Table 1), RB (designated **11** in Table 1), APC (designated **12** in Table 1), DCC (designated **13** in Table 1), NF-1 (designated **14** in Table 1), NF-2 (designated **15** in Table 1), WT-1 (designated **16** in Table 1), MEN-I (designated **17** in Table 1), MEN-II (designated **18** in Table 1), zac1 (designated **19** in Table 1), p73 (designated **20** in Table 1), VHL (designated **21** in Table 1), FCC (designated **23** in Table 1), MCC (designated **24** in Table 1), DBCCR1 (designated **133** in Table 1), DCP4 (designated **137** in Table 1) and p57 (designated **138** in Table 1).

B. Inducers of Apoptosis

Inducers of apoptosis, such as Bax (designated **25** in Table 1), Bak (designated **26** in Table 1), Bcl-X_s (designated **27** in Table 1), Bad (designated **28** in Table 1), Bim (designated **29** in Table 1), Bik (designated **30** in Table 1), Bid (designated **31** in Table 1), Harakiri (designated **32** in Table 1), Ad E1B (designated **33** in Table 1), Bad (designated **34** in Table 1), ICE-CED3 proteases (designated **35** in Table 1), TRAIL (designated **125** in Table 1), SARP-2 (designated **126** in Table 1) and apoptin (designated **132** in Table 1), similarly could find use according to the present invention. In addition, the delivery and regulated expression of cytotoxic genes have been described in the U.S. Patent Application entitled, "Induction of Apoptic or Cytotoxic Gene Expression by

Adenoviral Mediated Gene Codelivery," filed March 11, 1999 (specifically incorporated herein by reference).

C. Enzymes

Various enzyme genes are of interest according to the present invention. Such enzymes include cytosine deaminase (designated **36** in Table 1), adenosine deaminase (designated **37** in Table 1), hypoxanthine-guanine phosphoribosyltransferase (designated **38** in Table 1), galactose-1-phosphate uridyltransferase (designated **39** in Table 1), phenylalanine hydroxylase (designated **40** in Table 1), glucocerbrosidase (designated **41** in Table 1), sphingomyelinase (designated **42** in Table 1), α-L-iduronidase (designated **43** in Table 1), glucose-6-phosphate dehydrogenase (designated **44** in Table 1), HSV thymidine kinase (designated **45** in Table 1) and human thymidine kinase (designated **46** in Table 1) and extracellular proteins such as collagenase (designated **118** in Table 1), matrix metalloprotease (designated **119** in Table 1), RSKB (designated **128** in Table 1), RSK1 (designated **129** in Table 1), RSK2 (designated **130** in Table 1), RSK3 (designated **131** in Table 1), thrombospondin (designated **134** in Table 1), fibronectin (designated **135** in Table 1) and plasminogen (designated **136** in Table 1). In other embodiments of the present invention, the use of anti-angiogenic factors are contemplated.

D. Cytokines

Another class of genes that is contemplated to be inserted into the adenoviral vectors of the present invention include interleukins and cytokines. Interleukin 1 (IL-1; designated **47** in Table 1), IL-2 (designated **48** in Table 1), IL-3 (designated **49** in Table 1), IL-4 (designated **50** in Table 1), IL-5 (designated **51** in Table 1), IL-6 (designated **52** in Table 1), IL-7 (designated **53** in Table 1), IL-8 (designated **54** in Table 1), IL-9 (designated **55** in Table 1), IL-10 (designated **56** in Table 1), IL-11 (designated **57** in Table 1), IL-12 (designated **58** in Table 1), IL-13 (designated **59** in Table 1), IL-14 (designated **60** in Table 1), IL-15 (designated **61** in Table 1), β-interferon (designated **62** in Table 1), α-interferon (designated **63** in Table 1), γ-interferon (designated **122** in Table 1), angiostatin (designated **64** in Table 1), thrombospondin (designated **65** in Table 1),

endostatin (designated **66** in Table 1), METH-1 (designated **67** in Table 1), METH-2 (designated **68** in Table 1), GM-CSF (designated **69** in Table 1), G-CSF (designated **70** in Table 1), M-CSF (designated **123** in Table 1) and tumor necrosis factor (designated **124** in Table 1).

E. Toxins

Various toxins are also contemplated to be useful as part of the expression vectors of the present invention, these toxins include bacterial toxins such as ricin A-chain (Burbage, 1997; designated **71** in Table 1), diphtheria toxin A (Massuda *et al.*, 1997; Lidor, 1997; designated **72** in Table 1), pertussis toxin A subunit (designated **73** in Table 1), *E. coli* enterotoxin toxin A subunit (designated **74** in Table 1), cholera toxin A subunit (designated **75** in Table 1) and pseudomonas toxin c-terminal (designated **76** in Table 1). Recently, it was demonstrated that transfection of a plasmid containing the fusion protein regulatable diphtheria toxin A chain gene was cytotoxic for cancer cells. Thus, gene transfer of regulated toxin genes might also be applied to the treatment of cancers (Massuda *et al.*, 1997).

F. Antisense Constructs

Antisense methodology takes advantage of the fact that nucleic acids tend to pair with "complementary" sequences. By complementary, it is meant that polynucleotides are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, the larger purines will base pair with the smaller pyrimidines to form combinations of guanine paired with cytosine (G:C) and adenine paired with either thymine (A:T) in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA. Inclusion of less common bases such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine and others in hybridizing sequences does not interfere with pairing.

Targeting double-stranded (ds) DNA with polynucleotides leads to triple-helix formation; targeting RNA will lead to double-helix formation. Antisense

polynucleotides, when introduced into a target cell, specifically bind to their target polynucleotide and interfere with transcription, RNA processing, transport, translation and/or stability. Antisense RNA constructs, or DNA encoding such antisense RNA's, may be employed to inhibit gene transcription or translation or both within a host cell, either *in vitro* or *in vivo*, such as within a host animal, including a human subject.

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. It is contemplated that the most effective antisense constructs will include regions complementary to intron/exon splice junctions. Thus, it is proposed that a preferred embodiment includes an antisense construct with complementarity to regions within 50-200 bases of an intron-exon splice junction. It has been observed that some exon sequences can be included in the construct without seriously affecting the target selectivity thereof. The amount of exonic material included will vary depending on the particular exon and intron sequences used. One can readily test whether too much exon DNA is included simply by testing the constructs *in vitro* to determine whether normal cellular function is affected or whether the expression of related genes having complementary sequences is affected.

As stated above, "complementary" or "antisense" means polynucleotide sequences that are substantially complementary over their entire length and have very few base mismatches. For example, sequences of fifteen bases in length may be termed complementary when they have complementary nucleotides at thirteen or fourteen positions. Naturally, sequences which are completely complementary will be sequences which are entirely complementary throughout their entire length and have no base mismatches. Other sequences with lower degrees of homology also are contemplated. For example, an antisense construct which has limited regions of high homology, but also contains a non-homologous region (*e.g.*, ribozyme; see below) could be designed. These molecules, though having less than 50% homology, would bind to target sequences under appropriate conditions.

It may be advantageous to combine portions of genomic DNA with cDNA or synthetic sequences to generate specific constructs. For example, where an intron is desired in the ultimate construct, a genomic clone will need to be used. The cDNA or a synthesized polynucleotide may provide more convenient restriction sites for the remaining portion of the construct and, therefore, would be used for the rest of the sequence.

Particular oncogenes that are targets for antisense constructs are *ras* (designated **77** in Table 1), *myc* (designated **78** in Table 1), *neu* (designated **79** in Table 1), *raf* (designated **80** in Table 1), *erb* (designated **81** in Table 1), *src* (designated **82** in Table 1), *fms* (designated **83** in Table 1), *jun* (designated **84** in Table 1), *trk* (designated **85** in Table 1), *ret* (designated **86** in Table 1), *hst* (designated **87** in Table 1), *gsp* (designated **88** in Table 1), *bcl-2* (designated **89** in Table 1) and *abl* (designated **90** in Table 1). Also contemplated to be useful will be anti-apoptotic genes and angiogenesis promoters.

G. Ribozymes

Although proteins traditionally have been used for catalysis of nucleic acids, another class of macromolecules has emerged as useful in this endeavor. Ribozymes are RNA-protein complexes that cleave nucleic acids in a site-specific fashion. Ribozymes have specific catalytic domains that possess endonuclease activity (Kim and Cook, 1987; Gerlach *et al.*, 1987; Forster and Symons, 1987). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cook *et al.*, 1981; Michel and Westhof, 1990; Reinhold-Hurek and Shub, 1992). This specificity has been attributed to the requirement that the substrate bind *via* specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

Ribozyme catalysis has primarily been observed as part of sequence-specific cleavage/ligation reactions involving nucleic acids (Joyce, 1989; Cook *et al.*, 1981). For

example, U.S. Patent No. 5,354,855 reports that certain ribozymes can act as endonucleases with a sequence specificity greater than that of known ribonucleases and approaching that of the DNA restriction enzymes. Thus, sequence-specific ribozyme-mediated inhibition of gene expression may be particularly suited to therapeutic applications (Scanlon *et al.*, 1991; Sarver *et al.*, 1990). Recently, it was reported that ribozymes elicited genetic changes in some cells lines to which they were applied; the altered genes included the oncogenes H-ras, c-fos and genes of HIV. Most of this work involved the modification of a target mRNA, based on a specific mutant codon that is cleaved by a specific ribozyme. Targets for this embodiment will include angiogenic genes such as VEGFs and angiopoeiteins as well as the oncogenes (*e.g.*, *ras, myc, neu, raf, erb, src, fms, jun, trk, ret, hst, gsp, bcl-2, EGFR, grb2* and *abl*).

H. Single Chain Antibodies

In yet another embodiment, one gene may comprise a single-chain antibody. Methods for the production of single-chain antibodies are well known to those of skill in the art. The skilled artisan is referred to U.S. Patent No. 5,359,046, (incorporated herein by reference) for such methods. A single chain antibody is created by fusing together the variable domains of the heavy and light chains using a short peptide linker, thereby reconstituting an antigen binding site on a single molecule.

Single-chain antibody variable fragments (scFvs) in which the C-terminus of one variable domain is tethered to the N-terminus of the other *via* a 15 to 25 amino acid peptide or linker, have been developed without significantly disrupting antigen binding or specificity of the binding (Bedzyk *et al.*, 1990; Chaudhary *et al.*, 1990). These Fvs lack the constant regions (Fc) present in the heavy and light chains of the native antibody.

Antibodies to a wide variety of molecules are contemplated, such as oncogenes, growth factors, hormones, enzymes, transcription factors or receptors. Also contemplated are secreted antibodies, targeted to serum, against angiogenic factors (VEGF/VSP designated **91** in Table 1; βFGF designated **92** in Table 1; αFGF designated **93** in Table

1; others) and endothelial antigens necessary for angiogenesis (*i.e.*, V3 integrin, designated **94** in Table 1). Specifically contemplated are growth factors such as transforming growth factor (designated **120** in Table 1) and platelet derived growth factor (designated **121** in Table 1).

I. Transcription Factors and Regulators

Another class of genes that can be applied in an advantageous combination are transcription factors. Examples include C/EBPα (designated **95** in Table 1), IκB (designated **96** in Table 1), NfκB (designated **97** in Table 1), Par-4 (designated **98** in Table 1) and C/EBPα (designated **127** in Table 1)

J. Cell Cycle Regulators

Cell cycle regulators provide possible advantages, when combined with other genes. Such cell cycle regulators include p27 (designated **99** in Table 1), p16 (designated **100** in Table 1), p21 (designated **4** in Table 1), p57 (designated **101** in Table 1), p18 (designated **102** in Table 1), p73 (designated **103** in Table 1), p19 (designated **104** in Table 1), p15 (designated **5** in Table 1), E2F-1 (designated **105** in Table 1), E2F-2 (designated **106** in Table 1), E2F-3 (designated **107** in Table 1), p107 (designated **109** in Table 1), p130 (designated **110** in Table 1) and E2F-4 (designated **111** in Table 1). Other cell cycle regulators include anti-angiogenic proteins, such as soluble Flt1 (dominant negative soluble VEGF receptor; designated **112** in Table 1), soluble Wnt receptors (designated **113** in Table 1), soluble Tie2/Tek receptor (designated **114** in Table 1), soluble hemopexin domain of matrix metalloprotease 2 (designated **115** in Table 1) and soluble receptors of other angiogenic cytokines (*e.g.* VEGFR1/KDR (designated **116** in Table 1), VEGFR3/Flt4 (designated **117** in Table 1), both VEGF receptors).

K. Chemokines

Genes that code for chemokines also may be used in the present invention. Chemokines generally act as chemoattractants to recruit immune effector cells to the site of chemokine expression. It may be advantageous to express a particular chemokine gene

in combination with, for example, a cytokine gene, to enhance the recruitment of other immune system components to the site of treatment. Such chemokines include RANTES (designated **10** in Table 1), MCAF, MIP1-alpha (designated **108** in Table 1), MIP1-Beta (designated **22** in Table 1), and IP-10. The skilled artisan will recognize that certain cytokines are also known to have chemoattractant effects and could also be classified under the term chemokines.

L. Gene Combinations

As described herein, it is contemplated that any one particular gene may be combined with any other particular gene. Particularly preferred examples of gene combinations are listed in Table 1 herein below. Thus, any gene in the first column of Table 1 may be advantageously combined with any other gene depicted in the first row of Table 1. Of course these are only exemplary combinations and given the teachings of the present invention one of skill in the art will be able to produce multigene constructs of any conceivable combination, including but not limited to combinations two, three, four, five or more genes or nucleic acid constructs. The following graphic may be helpful to one of ordinary skill in the art viewing Table 1. This Table is split into 36 leaves labeled consecutively leaf a, leaf b, leaf c, leaf d, leaf e, leaf f, leaf g, leaf h, leaf i, leaf j, leaf k, leaf l, leaf m, leaf n, leaf o, leaf p, leaf q, leaf r, leaf s, leaf t, leaf u, leaf v, leaf w, leaf x, leaf y, leaf z, leaf aa, leaf bb, leaf cc, leaf dd, leaf ee, leaf ff, leaf gg, leaf hh, leaf ii and leaf jj; in order to view the Table as a whole the leaves are arranged in the following spatial order:

a	**b**	**c**	**d**	**e**	**f**	**g**	**h**	**hh**
i	**k**	**m**	**o**	**q**	**s**	**u**	**w**	**ii**
j	**l**	**n**	**p**	**r**	**t**	**v**	**x**	**jj**
y	**z**	**aa**	**bb**	**cc**	**dd**	**ee**	**ff**	**gg**

Thus, Table 1 has been split into separate pages in order to conform to the specification however it is under stood that Row 1 of table 1 contains the individual numbers from 1 through to 140 (inclusive) and Column 1 of Table 1 contains the

individual numbers from 1 through to 140 (inclusive). These numerals refer to individual genes as designated throughout the specification for example numeral 1 refers to p53, numeral 2 refers to C-CAM, numeral 3 refers to CEA, numeral 4 refers to p21, numeral 5 refers to p15, numeral 6 refers to BRCA1. Each box marked with an "X" denotes a combination comprising the gene located in that horizontal row and with the gene located in the vertical column. Given the format of this Table it should be easy for one of skill in the art to add further genes to this table to work out possible combinations contemplated.

Table 1 (leaf a) Specific Gene Combinations.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1		X	X	X	X	X	X	X	X	X	X	X	X	X	X
2	X		X	X	X	X	X	X	X	X	X	X	X	X	X
3	X	X		X	X	X	X	X	X	X	X	X	X	X	X
4	X	X	X		X	X	X	X	X	X	X	X	X	X	X
5	X	X	X	X		X	X	X	X	X	X	X	X	X	X
6	X	X	X	X	X		X	X	X	X	X	X	X	X	X
7	X	X	X	X	X	X		X	X	X	X	X	X	X	X
8	X	X	X	X	X	X	X		X	X	X	X	X	X	X
9	X	X	X	X	X	X	X	X		X	X	X	X	X	X
10	X	X	X	X	X	X	X	X	X		X	X	X	X	X
11	X	X	X	X	X	X	X	X	X	X		X	X	X	X
12	X	X	X	X	X	X	X	X	X	X	X		X	X	X
13	X	X	X	X	X	X	X	X	X	X	X	X		X	X
14	X	X	X	X	X	X	X	X	X	X	X	X	X		X
15	X	X	X	X	X	X	X	X	X	X	X	X	X	X	
16	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
17	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
18	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
19	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
20	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
21	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
22	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
23	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
24	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
25	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
26	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
27	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
28	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
29	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
30	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
31	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
32	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
33	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
34	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
35	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
36	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
37	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
38	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
39	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
40	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf b)

	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
3	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
4	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
5	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
6	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
7	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
8	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
9	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
10	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
11	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
12	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
13	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
14	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
15	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
16		X	X	X	X	X	X	X	X	X	X	X	X	X	X
17	X		X	X	X	X	X	X	X	X	X	X	X	X	X
18	X	X		X	X	X	X	X	X	X	X	X	X	X	X
19	X	X	X		X	X	X	X	X	X	X	X	X	X	X
20	X	X	X	X		X	X	X	X	X	X	X	X	X	X
21	X	X	X	X	X		X	X	X	X	X	X	X	X	X
22	X	X	X	X	X	X		X	X	X	X	X	X	X	X
23	X	X	X	X	X	X	X		X	X	X	X	X	X	X
24	X	X	X	X	X	X	X	X		X	X	X	X	X	X
25	X	X	X	X	X	X	X	X	X		X	X	X	X	X
26	X	X	X	X	X	X	X	X	X	X		X	X	X	X
27	X	X	X	X	X	X	X	X	X	X	X		X	X	X
28	X	X	X	X	X	X	X	X	X	X	X	X		X	X
29	X	X	X	X	X	X	X	X	X	X	X	X	X		X
30	X	X	X	X	X	X	X	X	X	X	X	X	X	X	
31	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
32	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
33	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
34	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
35	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
36	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
37	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
38	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
39	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
40	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf c)

	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
3	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
4	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
5	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
6	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
7	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
8	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
9	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
10	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
11	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
12	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
13	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
14	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
15	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
16	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
17	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
18	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
19	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
20	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
21	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
22	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
23	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
24	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
25	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
26	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
27	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
28	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
29	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
30	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
31		X	X	X	X	X	X	X	X	X	X	X	X	X	X
32	X		X	X	X	X	X	X	X	X	X	X	X	X	X
33	X	X		X	X	X	X	X	X	X	X	X	X	X	X
34	X	X	X		X	X	X	X	X	X	X	X	X	X	X
35	X	X	X	X		X	X	X	X	X	X	X	X	X	X
36	X	X	X	X	X		X	X	X	X	X	X	X	X	X
37	X	X	X	X	X	X		X	X	X	X	X	X	X	X
38	X	X	X	X	X	X	X		X	X	X	X	X	X	X
39	X	X	X	X	X	X	X	X		X	X	X	X	X	X
40	X	X	X	X	X	X	X	X	X		X	X	X	X	X

Table 1 (Continued; leaf d)

	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
3	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
4	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
5	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
6	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
7	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
8	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
9	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
10	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
11	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
12	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
13	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
14	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
15	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
16	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
17	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
18	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
19	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
20	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
21	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
22	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
23	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
24	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
25	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
26	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
27	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
28	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
29	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
30	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
31	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
32	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
33	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
34	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
35	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
36	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
37	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
38	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
39	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
40	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf e)

	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
3	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
4	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
5	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
6	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
7	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
8	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
9	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
10	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
11	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
12	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
13	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
14	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
15	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
16	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
17	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
18	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
19	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
20	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
21	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
22	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
23	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
24	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
25	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
26	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
27	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
28	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
29	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
30	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
31	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
32	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
33	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
34	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
35	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
36	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
37	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
38	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
39	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
40	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf f)

	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
3	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
4	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
5	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
6	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
7	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
8	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
9	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
10	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
11	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
12	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
13	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
14	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
15	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
16	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
17	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
18	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
19	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
20	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
21	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
22	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
23	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
24	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
25	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
26	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
27	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
28	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
29	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
30	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
31	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
32	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
33	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
34	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
35	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
36	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
37	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
38	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
39	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
40	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf g)

	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107
1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
3	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
4	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
5	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
6	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
7	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
8	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
9	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
10	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
11	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
12	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
13	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
14	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
15	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
16	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
17	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
18	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
19	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
20	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
21	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
22	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
23	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
24	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
25	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
26	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
27	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
28	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
29	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
30	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
31	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
32	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
33	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
34	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
35	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
36	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
37	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
38	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
39	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
40	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf h)

	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124
1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
3	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
4	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
5	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
6	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
7	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
8	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
9	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
10	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
11	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
12	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
13	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
14	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
15	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
16	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
17	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
18	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
19	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
20	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
21	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
22	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
23	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
24	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
25	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
26	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
27	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
28	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
29	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
30	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
31	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
32	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
33	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
34	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
35	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
36	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
37	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
38	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
39	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
40	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf i)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
41	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
42	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
43	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
44	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
45	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
46	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
47	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
48	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
49	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
50	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
51	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
52	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
53	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
54	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
55	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
56	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
57	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
58	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
59	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
60	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
61	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
62	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
63	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
64	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
65	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
66	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
67	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
68	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
69	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
70	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
71	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
72	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
73	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
74	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
75	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
76	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
77	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
78	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
79	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
80	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf j)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
81	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
82	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
83	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
84	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
85	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
86	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
87	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
88	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
89	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
90	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
91	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
92	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
93	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
94	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
95	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
96	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
97	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
98	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
99	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
100	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
101	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
102	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
103	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
104	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
105	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
106	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
107	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
108	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
109	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
110	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
111	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
112	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
113	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
114	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
115	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
116	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
117	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
118	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
119	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
120	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
121	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
122	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
123	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
124	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf k)

	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
41	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
42	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
43	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
44	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
45	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
46	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
47	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
48	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
49	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
50	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
51	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
52	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
53	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
54	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
55	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
56	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
57	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
58	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
59	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
60	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
61	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
62	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
63	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
64	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
65	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
66	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
67	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
68	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
69	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
70	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
71	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
72	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
73	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
74	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
75	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
76	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
77	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
78	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
79	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
80	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf l)

	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
81	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
82	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
83	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
84	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
85	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
86	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
87	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
88	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
89	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
90	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
91	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
92	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
93	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
94	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
95	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
96	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
97	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
98	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
99	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
100	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
101	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
102	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
103	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
104	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
105	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
106	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
107	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
108	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
109	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
110	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
111	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
112	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
113	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
114	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
115	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
116	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
117	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
118	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
119	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
120	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
121	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
122	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
123	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
124	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf m)

	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
41	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
42	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
43	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
44	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
45	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
46	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
47	X	X	X	X	X	X	X	X	X	X	X	X	X	X	*
48	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
49	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
50	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
51	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
52	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
53	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
54	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
55	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
56	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
57	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
58	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
59	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
60	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
61	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
62	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
63	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
64	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
65	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
66	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
67	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
68	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
69	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
70	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
71	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
72	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
73	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
74	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
75	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
76	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
77	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
78	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
79	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
80	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf n)

	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
81	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
82	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
83	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
84	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
85	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
86	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
87	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
88	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
89	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
90	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
91	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
92	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
93	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
94	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
95	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
96	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
97	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
98	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
99	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
100	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
101	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
102	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
103	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
104	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
105	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
106	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
107	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
108	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
109	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
110	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
111	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
112	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
113	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
114	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
115	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
116	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
117	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
118	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
119	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
120	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
121	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
122	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
123	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
124	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf o)

	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
41	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
42	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
43	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
44	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
45	X	X	*	X	X	X	X	X	X	X	X	X	X	X	X
46		X	X	X	X	X	X	X	X	X	X	X	X	X	X
47	X		X	X	X	X	X	X	X	X	X	X	X	X	X
48	X	X		X	X	X	X	X	X	X	X	X	X	X	X
49	X	X	X		X	X	X	X	X	X	X	X	X	X	X
50	X	X	X	X		X	X	X	X	X	X	X	X	X	X
51	X	X	X	X	X		X	X	X	X	X	X	X	X	X
52	X	X	X	X	X	X		X	X	X	X	X	X	X	X
53	X	X	X	X	X	X	X		X	X	X	X	X	X	X
54	X	X	X	X	X	X	X	X		X	X	X	X	X	X
55	X	X	X	X	X	X	X	X	X		X	X	X	X	X
56	X	X	X	X	X	X	X	X	X	X		X	X	X	X
57	X	X	X	X	X	X	X	X	X	X	X		X	X	X
58	X	X	X	X	X	X	X	X	X	X	X	X		X	X
59	X	X	X	X	X	X	X	X	X	X	X	X	X		X
60	X	X	X	X	X	X	X	X	X	X	X	X	X	X	
61	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
62	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
63	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
64	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
65	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
66	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
67	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
68	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
69	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
70	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
71	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
72	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
73	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
74	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
75	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
76	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
77	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
78	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
79	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
80	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf p)

	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
81	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
82	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
83	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
84	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
85	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
86	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
87	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
88	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
89	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
90	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
91	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
92	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
93	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
94	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
95	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
96	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
97	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
98	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
99	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
100	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
101	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
102	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
103	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
104	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
105	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
106	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
107	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
108	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
109	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
110	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
111	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
112	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
113	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
114	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
115	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
116	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
117	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
118	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
119	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
120	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
121	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
122	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
123	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
124	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf q)

	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
41	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
42	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
43	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
44	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
45	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
46	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
47	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
48	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
49	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
50	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
51	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
52	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
53	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
54	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
55	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
56	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
57	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
58	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
59	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
60	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
61		X	X	X	X	X	X	X	X	X	X	X	X	X	X
62	X		X	X	X	X	X	X	##	X	X	X	X	X	X
63	X	X		X	X	X	X	X	#	X	X	X	X	X	X
64	X	X	X		X	X	X	X	X	X	X	X	X	X	X
65	X	X	X	X		X	X	X	X	X	X	X	X	X	X
66	X	X	X	X	X		X	X	X	X	X	X	X	X	X
67	X	X	X	X	X	X		X	X	X	X	X	X	X	X
68	X	X	X	X	X	X	X		X	X	X	X	X	X	X
69	X	##	#	X	X	X	X	X		X	X	X	X	X	X
70	X	X	X	X	X	X	X	X	X		X	X	X	X	X
71	X	X	X	X	X	X	X	X	X	X		X	X	X	X
72	X	X	X	X	X	X	X	X	X	X	X		X	X	X
73	X	X	X	X	X	X	X	X	X	X	X	X		X	X
74	X	X	X	X	X	X	X	X	X	X	X	X	X		X
75	X	X	X	X	X	X	X	X	X	X	X	X	X	X	
76	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
77	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
78	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
79	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
80	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf r)

	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
81	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
82	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
83	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
84	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
85	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
86	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
87	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
88	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
89	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
90	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
91	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
92	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
93	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
94	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
95	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
96	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
97	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
98	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
99	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
100	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
101	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
102	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
103	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
104	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
105	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
106	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
107	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
108	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
109	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
110	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
111	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
112	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
113	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
114	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
115	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
116	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
117	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
118	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
119	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
120	X	X	X	X	X	X	X	X	Ψ	X	X	X	X	X	X
121	X	X	X	X	X	X	X	X	ϕ	X	X	X	X	X	X
122	X	X	X	X	X	X	X	X	φ	X	X	X	X	X	X
123	X	X	X	X	X	X	X	X	θ	X	X	X	X	X	X
124	X	X	X	X	X	X	X	X	ρ	X	X	X	X	X	X

Table 1 (Continued; leaf s)

	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
41	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
42	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
43	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
44	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
45	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
46	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
47	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
48	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
49	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
50	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
51	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
52	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
53	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
54	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
55	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
56	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
57	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
58	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
59	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
60	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
61	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
62	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
63	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
64	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
65	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
66	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
67	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
68	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
69	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
70	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
71	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
72	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
73	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
74	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
75	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
76		X	X	X	X	X	X	X	X	X	X	X	X	X	X
77	X		X	X	X	X	X	X	X	X	X	X	X	X	X
78	X	X		X	X	X	X	X	X	X	X	X	X	X	X
79	X	X	X		X	X	X	X	X	X	X	X	X	X	X
80	X	X	X	X		X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf t)

	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
81	X	X	X	X	X		X	X	X	X	X	X	X	X	X
82	X	X	X	X	X	X		X	X	X	X	X	X	X	X
83	X	X	X	X	X	X	X		X	X	X	X	X	X	X
84	X	X	X	X	X	X	X	X		X	X	X	X	X	X
85	X	X	X	X	X	X	X	X	X		X	X	X	X	X
86	X	X	X	X	X	X	X	X	X	X		X	X	X	X
87	X	X	X	X	X	X	X	X	X	X	X		X	X	X
88	X	X	X	X	X	X	X	X	X	X	X	X		X	X
89	X	X	X	X	X	X	X	X	X	X	X	X	X		X
90	X	X	X	X	X	X	X	X	X	X	X	X	X	X	
91	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
92	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
93	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
94	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
95	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
96	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
97	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
98	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
99	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
100	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
101	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
102	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
103	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
104	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
105	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
106	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
107	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
108	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
109	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
110	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
111	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
112	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
113	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
114	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
115	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
116	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
117	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
118	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
119	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
120	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
121	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
122	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
123	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
124	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf u)

	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108
41	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
42	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
43	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
44	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
45	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
46	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
47	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
48	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
49	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
50	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
51	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
52	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
53	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
54	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
55	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
56	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
57	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
58	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
59	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
60	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
61	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
62	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
63	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
64	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
65	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
66	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
67	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
68	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
69	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
70	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
71	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
72	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
73	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
74	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
75	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
76	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
77	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
78	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
79	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
80	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf v)

	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107
81	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
82	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
83	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
84	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
85	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
86	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
87	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
88	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
89	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
90	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
91		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
92	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
93	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X
94	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X
95	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X
96	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X
97	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X
98	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X
99	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X
100	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X
101	X	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X
102	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X	X
103	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X
104	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X
105	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X
106	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X
107	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	
108	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
109	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
110	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
111	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
112	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
113	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
114	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
115	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
116	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
117	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
118	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
119	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
120	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
121	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
122	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
123	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
124	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf w)

	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124
41	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
42	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
43	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
44	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
45	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
46	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
47	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
48	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
49	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
50	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
51	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
52	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
53	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
54	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
55	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
56	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
57	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
58	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
59	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
60	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
61	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
62	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
63	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
64	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
65	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
66	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
67	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
68	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
69	X	X	X	X	X	X	X	X	X	X	X	Ψ	ϕ	φ	θ	ρ
70	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
71	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
72	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
73	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
74	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
75	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
76	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
77	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
78	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
79	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
80	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf x)

	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124
81	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
82	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
83	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
84	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
85	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
86	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
87	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
88	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
89	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
90	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
91	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
92	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
93	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
94	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
95	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
96	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
97	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
98	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
99	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
100	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
101	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
102	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
103	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
104	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
105	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
106	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
107	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
108		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
109	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
110	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X
111	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X
112	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X
113	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X
114	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X
115	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X
116	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X
117	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X
118	X	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X
119	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X	X
120	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X
121	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X
122	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X
123	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X
124	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	

Table 1 (Continued; leaf y)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
125	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
126	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
127	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
128	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
129	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
130	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
131	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
132	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
133	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
134	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
135	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
136	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
137	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
138	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
139	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
140	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf z)

	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
125	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
126	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
127	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
128	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
129	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
130	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
131	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
132	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
133	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
134	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
135	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
136	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
137	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
138	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
139	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
140	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf aa)

	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
125	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
126	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
127	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
128	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
129	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
130	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
131	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
132	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
133	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
134	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
135	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
136	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
137	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
138	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
139	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
140	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf bb)

	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
125	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
126	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
127	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
128	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
129	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
130	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
131	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
132	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
133	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
134	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
135	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
136	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
137	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
138	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
139	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
140	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf cc)

	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
125	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
126	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
127	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
128	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
129	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
130	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
131	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
132	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
133	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
134	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
135	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
136	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
137	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
138	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
139	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
140	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf dd)

	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
125	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
126	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
127	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
128	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
129	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
130	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
131	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
132	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
133	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
134	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
135	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
136	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
137	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
138	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
139	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
140	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf ee)

	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107
125	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
126	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
127	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
128	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
129	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
130	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
131	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
132	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
133	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
134	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
135	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
136	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
137	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
138	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
139	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
140	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf ff)

	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124
125	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
126	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
127	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
128	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
129	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
130	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
131	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
132	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
133	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
134	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
135	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
136	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
137	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
138	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
139	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
140	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf gg)

	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140
125		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
126	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X
127	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X
128	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X
129	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X
130	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X
131	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X
132	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X
133	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X
134	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X
135	X	X	X	X	X	X	X	X	X	X		X	X	X	X	X
136	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X
137	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X
138	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X
139	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X
140	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	

Table 1 (Continued; leaf hh)

	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140
1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
3	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
4	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
5	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
6	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
7	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
8	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
9	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
10	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
11	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
12	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
13	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
14	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
15	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
16	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
17	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
18	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
19	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
20	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
21	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
22	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
23	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
24	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
25	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
26	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
27	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
28	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
29	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
30	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
31	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
32	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
33	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
34	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
35	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
36	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
37	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
38	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
39	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
40	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf ii)

	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140
41	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
42	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
43	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
44	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
45	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
46	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
47	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
48	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
49	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
50	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
51	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
52	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
53	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
54	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
55	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
56	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
57	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
58	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
59	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
60	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
61	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
62	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
63	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
64	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
65	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
66	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
67	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
68	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
69	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
70	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
71	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
72	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
73	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
74	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
75	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
76	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
77	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
78	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
79	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
80	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Table 1 (Continued; leaf jj)

	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140
81	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
82	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
83	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
84	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
85	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
86	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
87	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
88	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
89	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
90	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
91	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
92	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
93	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
94	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
95	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
96	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
97	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
98	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
99	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
100	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
101	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
102	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
103	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
104	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
105	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
106	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
107	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
108	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
109	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
110	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
111	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
112	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
113	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
114	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
115	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
116	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
117	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
118	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
119	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
120	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
121	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
122	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
123	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
124	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

Footnotes pertaining to Table 1: * combination of IL-2 with HSV-tk in an adenovirus vector was described by O'Malley *et al.*, (1997 and 1996 both incorporated herein by reference). # denotes the combination of GM-CSF and IFNα described in WO 97/32481 (incorporated herein by reference). ## denotes the combination of GM-CSF and IFNβ described in WO 97/32481 (incorporated herein by reference). Ψ denotes the combination of GM-CSF and TGF as described in WO 97/32481 (incorporated herein by reference). ϕ denotes the combination of PDGF and GM-CSF as described in WO 97/32481 (incorporated herein by reference). φ denotes the combination of IFNγ and GM-CSF as described in WO 97/32481 (incorporated herein by reference). θ denotes the combination of M-CSF and GM-CSF as described in WO 97/32481 (incorporated herein by reference). ρ denotes the combination of tumor necrosis factor and GM-CSF as described in WO 97/32481 (incorporated herein by reference).

V. Regulatory Elements

A. Promoters

Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a nucleic acid coding for gene products in which part or all of the nucleic acid encoding sequence is capable of being transcribed. The transcript may be translated into a protein, but it need not be. In certain embodiments, expression includes both transcription of a gene and translation of mRNA into a gene product. In other embodiments, expression only includes transcription of the nucleic acid encoding genes of interest.

The nucleic acid encoding a gene product is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (*tk*) and SV40 early

transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter employed to control the expression of a nucleic acid sequence of interest is not believed to be important, so long as it is capable of directing the expression of the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

In various embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, β-

actin, rat insulin promoter, human ubiquitin C promoter and glyceraldehyde-3-phosphate dehydrogenase can be used to obtain high-level expression of the coding sequence of interest. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a coding sequence of interest is contemplated as well, provided that the levels of expression are sufficient for a given purpose. By employing a promoter with well-known properties, the level and pattern of expression of the protein of interest following transfection or transformation can be optimized.

Selection of a promoter that is regulated in response to specific physiologic or synthetic signals can permit inducible expression of the gene product. For example in the case where expression of a transgene, or transgenes when a multicistronic vector is utilized, is toxic to the cells in which the vector is produced in, it may be desirable to prohibit or reduce expression of one or more of the transgenes. Examples of transgenes that may be toxic to the producer cell line are pro-apoptotic and cytokine genes. Several inducible promoter systems are available for production of viral vectors where the transgene product may be toxic.

The ecdysone system (Invitrogen, Carlsbad, CA) is one such system. This system is designed to allow regulated expression of a gene of interest in mammalian cells. It consists of a tightly regulated expression mechanism that allows virtually no basal level expression of the transgene, but over 200-fold inducibility. The system is based on the heterodimeric ecdysone receptor of Drosophila, and when ecdysone or an analog such as muristerone A binds to the receptor, the receptor activates a promoter to turn on expression of the downstream transgene high levels of mRNA transcripts are attained. In this system, both monomers of the heterodimeric receptor are constitutively expressed from one vector, whereas the ecdysone-responsive promoter which drives expression of the gene of interest is on another plasmid. Engineering of this type of system into the gene transfer vector of interest would therefore be useful. Cotransfection of plasmids containing the gene of interest and the receptor monomers in the producer cell line would

then allow for the production of the gene transfer vector without expression of a potentially toxic transgene. At the appropriate time, expression of the transgene could be activated with ecdysone or muristeron A.

Another inducible system that would be useful is the Tet-Off™ or Tet-On™ system (Clontech, Palo Alto, CA) originally developed by Gossen and Bujard (Gossen and Bujard, 1992; Gossen *et al.*, 1995). This system also allows high levels of gene expression to be regulated in response to tetracycline or tetracycline derivatives such as doxycycline. In the Tet-On™ system, gene expression is turned on in the presence of doxycycline, whereas in the Tet-Off™ system, gene expression is turned on in the absence of doxycycline. These systems are based on two regulatory elements derived from the tetracycline resistance operon of *E. coli*. The tetracycline operator sequence to which the tetracycline repressor binds, and the tetracycline repressor protein. The gene of interest is cloned into a plasmid behind a promoter that has tetracycline-responsive elements present in it. A second plasmid contains a regulatory element called the tetracycline-controlled transactivator, which is composed, in the Tet-Off™ system, of the VP16 domain from the herpes simplex virus and the wild-type tetracycline repressor. Thus in the absence of doxycycline, transcription is constituitively on. In the Tet-On™ system, the tetracycline repressor is not wild type and in the presence of doxycycline activates transcription. For gene therapy vector production, the Tet-Off™ system would be preferable so that the producer cells could be grown in the presence of tetracycline or doxycycline and prevent expression of a potentially toxic transgene, but when the vector is introduced to the patient, the gene expression would be constituitively on.

In some circumstances, it may be desirable to regulate expression of a transgene in a gene therapy vector. For example, different viral promoters with varying strengths of activity may be utilized depending on the level of expression desired. In mammalian cells, the CMV immediate early promoter if often used to provide strong transcriptional activation. Modified versions of the CMV promoter that are less potent have also been used when reduced levels of expression of the transgene are desired. When expression of

a transgene in hematopoetic cells is desired, retroviral promoters such as the LTRs from MLV or MMTV are often used. Other viral promoters that may be used depending on the desired effect include SV40, RSV LTR, HIV-1 and HIV-2 LTR, adenovirus promoters such as from the E1A, E2A, or MLP region, AAV LTR, cauliflower mosaic virus, HSV-TK, and avian sarcoma virus.

Similarly tissue specific promoters may be used to effect transcription in specific tissues or cells so as to reduce potential toxicity or undesirable effects to non-targeted tissues. For example, promoters such as the PSA, probasin, prostatic acid phosphatase or prostate-specific glandular kallikrein (hK2) may be used to target gene expression in the prostate. Similarly, the following promoters may be used to target gene expression in other tissues (Table 2).

Table 2. Tissue specific promoters

Tissue	Promoter
Pancreas	insulin
	elastin
	amylase
	pdr-1 pdx-1
	glucokinase
Liver	albumin PEPCK
	HBV enhancer
	alpha fetoprotein
	apolipoprotein C
	alpha-1 antitrypsin
	vitellogenin, NF-AB
	Transthyretin
Skeletal muscle	myosin H chain
	muscle creatine kinase
	dystrophin
	calpain p94
	skeletal alpha-actin
	fast troponin 1
Skin	keratin K6
	keratin K1
Lung	CFTR
	human cytokeratin 18 (K18)
	pulmonary surfactant proteins A, B and C
	CC-10
	P1
Smooth muscle	sm22 alpha
	SM-alpha-actin
Endothelium	endothelin-1
	E-selectin
	von Willebrand factor
	TIE (Korhonen *et al.*, 1995)
	KDR/flk-1
Melanocytes	tyrosinase
Adipose tissue	lipoprotein lipase (Zechner *et al.*, 1988)
	adipsin (Spiegelman *et al.*, 1989)
	acetyl-CoA carboxylase (Pape and Kim, 1989)
	glycerophosphate dehydrogenase (Dani *et al.*, 1989)
	adipocyte P2 (Hunt *et al.*, 1986)
Blood	β-globin

In certain indications, it may be desirable to activate transcription at specific times after administration of the gene therapy vector. This may be done with such promoters as those that are hormone or cytokine regulatable. For example in gene therapy applications where the indication is a gonadal tissue where specific steroids are produced or routed to, use of androgen or estrogen regulated promoters may be advantageous. Such promoters that are hormone regulatable include MMTV, MT-1, ecdysone and RuBisco. Other hormone regulated promoters such as those responsive to thyroid, pituitary and adrenal hormones are expected to be useful in the present invention. Cytokine and inflammatory protein responsive promoters that could be used include K and T Kininogen (Kageyama *et al.*, 1987), c-fos, TNF-alpha, C-reactive protein (Arcone *et al.*, 1988), haptoglobin (Oliviero *et al.*, 1987), serum amyloid A2, C/EBP alpha, IL-1, IL-6 (Poli and Cortese, 1989), Complement C3 (Wilson *et al.*, 1990), IL-8, alpha-1 acid glycoprotein (Prowse and Baumann, 1988), alpha-1 antitypsin, lipoprotein lipase (Zechner *et al.*, 1988), angiotensinogen (Ron *et al.*, 1991), fibrinogen, c-jun (inducible by phorbol esters, TNF-alpha, UV radiation, retinoic acid, and hydrogen peroxide), collagenase (induced by phorbol esters and retinoic acid), metallothionein (heavy metal and glucocorticoid inducible), Stromelysin (inducible by phorbol ester, interleukin-1 and EGF), alpha-2 macroglobulin and alpha-1 antichymotrypsin.

It is envisioned that cell cycle regulatable promoters may be useful in the present invention. For example, in a bi-cistronic gene therapy vector, use of a strong CMV promoter to drive expression of a first gene such as p16 that arrests cells in the G1 phase could be followed by expression of a second gene such as p53 under the control of a promoter that is active in the G1 phase of the cell cycle, thus providing a "second hit" that would push the cell into apoptosis. Other promoters such as those of various cyclins, PCNA, galectin-3, E2F1, p53 and BRCA1 could be used.

Tumor specific promoters such as osteocalcin, hypoxia-responsive element (HRE), MAGE-4, CEA, alpha-fetoprotein, GRP78/BiP and tyrosinase may also be used

to regulate gene expression in tumor cells. Other promoters that could be used according to the present invention include Lac-regulatable, chemotherapy inducible (*e.g.* MDR), and heat (hyperthermia) inducible promoters, radiation-inducible (*e.g.*, EGR (Joki *et al.*, 1995)), Alpha-inhibin, RNA pol III tRNA met and other amino acid promoters, U1 snRNA (Bartlett *et al.*, 1996), MC-1, PGK, β-actin and α-globin. Many other promoters that may be useful are listed in Walther and Stein (1996).

It is envisioned that any of the above promoters alone or in combination with another may be useful according to the present invention depending on the action desired. In addition, this list of promoters is should not be construed to be exhaustive or limiting, those of skill in the art will know of other promoters that may be used in conjunction with the promoters and methods disclosed herein.

B. Enhancers

Enhancers are genetic elements that increase transcription from a promoter located at a distant position on the same molecule of DNA. Enhancers are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins. The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Below is a list of promoters additional to the tissue specific promoters listed above, cellular promoters/enhancers and inducible promoters/enhancers that could be used in combination with the nucleic acid encoding a gene of interest in an expression construct (Table 3 and Table 4). Additionally, any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of

the gene. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

TABLE 3

ENHANCER
Immunoglobulin Heavy Chain
Immunoglobulin Light Chain
T-Cell Receptor
HLA DQ α and DQ β
β-Interferon
Interleukin-2
Interleukin-2 Receptor
MHC Class II 5
MHC Class II HLA-DRα
β-Actin
Muscle Creatine Kinase
Prealbumin (Transthyretin)
Elastase *I*
Metallothionein
Collagenase
Albumin Gene
α-Fetoprotein
τ-Globin
β-Globin
e-fos
c-HA-ras
Insulin
Neural Cell Adhesion Molecule (NCAM)
α1-Antitrypsin
H2B (TH2B) Histone

ENHANCER
Mouse or Type I Collagen
Glucose-Regulated Proteins (GRP94 and GRP78)
Rat Growth Hormone
Human Serum Amyloid A (SAA)
Troponin I (TN I)
Platelet-Derived Growth Factor
Duchenne Muscular Dystrophy
SV40
Polyoma
Retroviruses
Papilloma Virus
Hepatitis B Virus
Human Immunodeficiency Virus
Cytomegalovirus
Gibbon Ape Leukemia Virus

TABLE 4

Element	Inducer
MT II	Phorbol Ester (TPA) Heavy metals
MMTV (mouse mammary tumor virus)	Glucocorticoids
ß-Interferon	poly(rI)X poly(rc)
Adenovirus 5 E2	Ela
c-jun	Phorbol Ester (TPA), H_2O_2
Collagenase	Phorbol Ester (TPA)
Stromelysin	Phorbol Ester (TPA), IL-1
SV40	Phorbol Ester (TPA)
Murine MX Gene	Interferon, Newcastle Disease Virus
GRP78 Gene	A23187
α-2-Macroglobulin	IL-6
Vimentin	Serum
MHC Class I Gene H-2kB	Interferon
HSP70	Ela, SV40 Large T Antigen
Proliferin	Phorbol Ester-TPA
Tumor Necrosis Factor	FMA
Thyroid Stimulating Hormone α Gene	Thyroid Hormone
Insulin E Box	Glucose

C. Polyadenylation Signals

Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed such as human or bovine growth

hormone and SV40 polyadenylation signals. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

D. IRES

In certain embodiments of the invention, the use of internal ribosome entry site (IRES) elements is contemplated to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5' methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picornavirus family (poliovirus and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message.

Any heterologous open reading frame can be linked to IRES elements. This includes genes for secreted proteins, multi-subunit proteins, encoded by independent genes, intracellular or membrane-bound proteins and selectable markers. In this way, expression of several proteins can be simultaneously engineered into a cell with a single construct and a single selectable marker.

VI. Methods for Producing Viral Particles

The traditional method for the generation of adenoviral particles is co-transfection followed by subsequent *in vivo* recombination of a shuttle plasmid (usually containing a small subset of the adenoviral genome and the gene of interest in an expression cassette) and an adenoviral helper plasmid (containing most of the entire adenoviral genome) into either 293 or 911 cells (obtained from Introgene, The Netherlands). After transfection,

the adenoviral plaques are isolated from the agarose overlaid cells and the viral particles are expanded for analysis. For detailed protocols the skilled artisan is referred to Graham and Prevac (1991).

Alternative technologies for the generation of novel adenoviral vectors (*i.e.*, vectors including the adenoviral genes necessary for 293 cell dependent viral replication and expression cassette(s) involving gene(s) of interest, include utilization of the bacterial artificial chromosome (BAC) system, *in vivo* bacterial recombination in a recA+ bacterial strain utilizing two plasmids containing complementary adenoviral sequences and expression cassettes, and the yeast artificial chromosome (YAC) system). Incorporated herein by reference are PCT publications 95/27071 and 96/33280 which provide details of adenoviral production methodologies. Methods for improved production and purification of adenoviral vectors have been described in U.S. Patent Application 08/975,519, filed November 20, 1997 (specifically incorporated herein by reference).

The following protocol provides an example of virus production and expansion used in the present invention. Of course this is only an exemplary protocol and one of ordinary skill in the art will be able to modify steps therein according to individual requirements.

A. Cotransfection of Adenoviral Plasmids to Make Adenovirus

Day 1:

In the morning, seed ATCC 293 cells at 1×10^6 cells per 60 mm dish

Day 2:

Using the protocol for calcium phosphate transfections, transfect 293 cells with 5µg of DNA per plate (2.5µg of each plasmid, *i.e.* pJM17 and shuttle vector).

-Transfect 2-3 plates each for the control (no DNA) and test plasmid:

-Transfect 1-2 plates with ß-gal to check efficiency.

Day 3:

Change media on cells in morning

In afternoon, trypsinize transfected plates, count cells, and seed at 1.5 - 1.75 × 10^6 cells per well of a 6-well dish.

Day 4:

X-gal stain β-gal plate to check efficiency.

Day 5:

Overlay transfected cells using procedures from SOP #TM001-04

Wait to see plaques (probably 4 - 7 days, but could be 3 - 4 wk when using YAC DNA)

B. Expansion of Adenovirus Following Plaque Purification

Day 1:

Seed a 24-well plate at 2 - 2.5 × 10^5 ATCC 293 cells/well two days prior to infection

Day 3:

Seed 60 mm dishes at 2 × 10^6 ATCC 293 cells/dish for infection on Day 5

Pick a plaque, using a sterile capillary pipette, into 150 µl of serum-free media

Vortex briefly to break up agarose

Aspirate media off 24-well plate

Infect with 100 µl of viral-containing media/well for 1 hr, rocking after 15 and 45 min

Mock infect control well with 100 µl of serum-free media

Add 1 ml of DMEM + 10% FBS

Wait to see CPE activity (about 48 h).

Day 5:

Seed 2 × 150 mm dishes (for each plaque isolated) at 1.2×10^7 ATCC 293 cells/dish for infection on Day 7 (or ~6×10^6 cells/dish for infection on Day 8).

After CPE appears in 24-well dish, harvest cells and supernatant into a 15 ml Corning tube.

Freeze/thaw 3X in liquid nitrogen and 37C H2O bath.

Spin at ~2000 rpm for 5 min in clinical centrifuge.

Filter supernatant through a 0.22 µm filter.

Aspirate media off 60 mm dish.

Infect with 550 µl of viral-containing media/60 mm dish for 1 hr, rocking after 15 and 45 min.

Mock infect control dish with 550 µl of serum-free media.

Add 5 ml DMEM + 10% FBS.

Wait to see CPE.

Day 7:

After CPE appears in 60 mm dish, harvest cells and supernatant into a 50 ml Corning tube.

Freeze/thaw 3X in liquid nitrogen and 37C H2O bath.

Spin at ~2000 rpm for 5 min in clinical centrifuge.

Filter supernatant through a 0.22 µm filter (reserve .5 - 1 ml for DNA isolation and PCR™).

Aspirate media off 150 mm dishes.

Infect with 2 ml of viral-containing media + 2 ml of serum-free media/150 mm dish for 1 hr, rocking after 15 and 45 min.

Mock infect control dish with 4 ml of serum-free media.

Add 20 ml DMEM + 10% FBS.

Wait to see CPE.

Day 9:

After CPE appears in 150 mm dishes, harvest cells and supernatant into 3 × 50 ml Corning tubes.

Freeze/thaw 3X in liquid nitrogen and 37C H2O bath.

Spin at ~2000 rpm for 5 min in clinical centrifuge.

Filter supernatant through a 0.22 μm filter into a sterile 50 ml Corning tube.

Viral lysate is ready to be titered.

VII. Disease States

The present invention deals with the treatment of disease states that involve hyperproliferative disorders including benign and malignant neoplasias. Such disorders include restinosis, cancer, multi-drug resistant cancer, primary, psoriasis, inflamatory bowel disease, rheumatoid arthritis, osteoarthritis and metastatic tumors.

In particular, the present invention is directed at the treatment of human cancers including cancers of the prostate, lung, brain, skin, liver, breast, lymphoid system, stomach, testicular, ovarian, pancreatic, bone, bone marrow, head and neck, cervical, esophagus, eye, gall bladder, kidney, adrenal glands, heart, colon, rectum and blood.

VIII. Screening For Anti-Tumor Activity In Multigene Adenoviral Constructs Using Animal Models

Animal models may be used as a screen for tumor suppressive effects of genes or gene combinations. Preferably, orthotopic animal models will be used so as to closely mimic the particular disease type being studied and to provide the most relevant results.

One type of orthotopic model is that for head and neck cancer, which involves the development of an animal model for the analysis of microscopic residual carcinomas and

microscopic seeding of body cavities. "Carcinoma," as used herein, may refer to a single cell or a multicellular tumor mass. In microscopic disease, the "tumor" will consist or one or a few carcinoma cells which cannot be observed with the naked eye. The animal model described herein is particularly advantageous mimicking (i) the post surgical environment of head and neck cancer patients, particularly in advanced stages of disease and (ii) the body cavity of an affected subject wherein microscopic carcinoma has been established. The model, similar to other animal models for cancer, derives from inoculation of tumor cells into an animal. A distinction, however, lies in the creation, subcutaneously, of a pouch that is a physiologic equivalent of a natural body cavity or a post-surgical cavity created by the excision of a tumor mass.

The instant invention preferably uses nude mice as the model organism. Virtually any animal may be employed, however, for use according to the present invention. Particularly preferred animals will be small mammals that are routinely used in laboratory protocols. Even more preferred animals will be those of the rodent group, such as mice, rats, guinea pigs and hamsters. Rabbits also are a preferred species. The criteria for choosing an animal will be largely dependent upon the particular preference of an investigator.

The first step is to create a tissue flap in the experimental animal. The term "tissue flap" means any incision in the flesh of the animal that exposes the target tissue. It is generally preferred that an incision be made in the dorsal flank of an animal, as this represents a readily accessible site. However, it will be understood that an incision could well be made at other points on the animal, and the choice of tissue sites may be dependent upon various factors such as the particular type of therapeutics that are being investigated.

Once a target tissue site is exposed, carcinoma cells, either individually or in microscopic tumors, are contacted with the tissue site. The most convenient manner for seeding the cancer cells into the tissue site is to apply a suspension of tissue culture media

containing the cells to the exposed tissue. Cancer cell application may be achieved simply using a sterile pipette or any other convenient applicator. Naturally, this procedure will be conducted under sterile conditions.

In a particular example, 2.5×10^6 cells are inoculated into the exposed tissue flap of a nude mouse. Those of skill in the art will be able to readily determine, for a given purpose, what the appropriate number of cells will be. The number of cells will be dependent upon various factors, such as the size of the animal, the site of incision, the replicative capacity of the tumor cells themselves, the time intended for tumor growth, the potential anti-tumor therapeutic to be tested, and the like. Although establishing an optimal model system for any particular type of tumor may require a certain adjustment in the number of cells administered, this in no way represents an undue amount of experimentation. Those skilled in the area of animal testing will appreciate that such optimization is required.

This can be accomplished, for example, by conducting preliminary studies in which differing numbers of cells are delivered to the animal and the cell growth is monitored following resealing of the tissue flap. Naturally, administering larger numbers of cells will result in a larger population of microscopic residual tumor cells.

In the present study the flaps were effectively sealed using mattress sutures. However, it is envisioned that persons skilled in the art may use any of a variety of methods routinely used to seal the incision such as the use of adhesives, clamps, stitches, sutures, *etc.*, depending on the particular use contemplated.

Other orthotopic animal models are well known in the art. The orthotopic lung cancer model, for example has been described in the literature. This protocol involves injection of tumor cells into the bronchus of a mouse wherein tumors will form in the bronchus and bronchioles, mimicking tumors commonly found in non-small cell lung

cancer patients. The skilled artisan will readily be able to adapt or modify each particular model for his intended purpose without undue experimentation.

IX. Treatment Protocols

Clinical protocols may be developed to facilitate the treatment of disease using the multigene constructs discussed herein and above. Patients may, but need not have received previous chemo-, radio- or gene therapies. Optimally, patients will have adequate bone marrow function (defined as peripheral absolute granulocyte count of > 2,000/mm^3 and platelet count of 100,000/mm^3), adequate liver function (bilirubin $\leq$ 1.5 mg/dl) and adequate renal function (creatinine < 1.5 mg/dl).

The protocol calls for single dose administration, *via* intratumoral injection, of a pharmaceutical composition containing between 10^6 and 10^9 infectious particles of the expression construct. For tumors of $\geq$ 4 cm, the volume administered will be 4-10 ml (preferably 10 ml), while for tumors < 4 cm, a volume of 1-3 ml will be used (preferably 3 ml). Multiple injections will be delivered for a single dose, in 0.1-0.5 ml volumes, with spacing of approximately 1 cm or more.

The treatment course consists of about six doses, delivered over two weeks. Upon election by the clinician, the regimen may be continued, six doses each two weeks, or on a less frequent (monthly, bimonthly, quarterly, *etc.*) basis.

Where patients are eligible for surgical resection, the tumor will be treated as described above for at least two consecutive two-week treatment courses. Within one wk of completion of the second (or more, *e.g.*, third, fourth, fifth, sixth, seventh, eighth, *etc.*) course, the patient will receive surgical resection. Prior to close of the incision, 10 ml of a pharmaceutical composition containing the expression construct (10^6-10^9 infectious particles) will be delivered to the surgical site (operative bed) and allowed to remain in contact for at least 60 min. The wound is closed and a drain or catheter placed therein. On the third post-operative day, additional 10 ml of the pharmaceutical composition is

administered *via* the drain and allowed to remain in contact with the operative bed for at least two h. Removal by suction is then performed, and the drain removed at a clinically appropriate time.

A. Treatment of Artificial and Natural Body Cavities

One of the prime sources of recurrent tumor growth is the residual, microscopic disease that remains at the primary tumor site, as well as locally and regionally, following tumor excision. In addition, there are analogous situations where natural body cavities are seeded by microscopic tumor cells. The effective treatment of such microscopic disease would present a significant advance in therapeutic regimens.

Thus, in certain embodiments, a cancer may be removed by surgical excision, creating a "cavity." Both at the time of surgery, and thereafter (periodically or continuously), the therapeutic composition of the present invention is administered to the body cavity. This is, in essence, a "topical" treatment of the surface of the cavity. The volume of the composition should be sufficient to ensure that the entire surface of the cavity is contacted by the expression construct.

In one embodiment, administration simply will entail injection of the therapeutic composition into the cavity formed by the tumor excision. In another embodiment, mechanical application *via* a sponge, swab or other device may be desired. Either of these approaches can be used subsequent to the tumor removal as well as during the initial surgery. In still another embodiment, a catheter is inserted into the cavity prior to closure of the surgical entry site. The cavity may then be continuously perfused for a desired period of time.

In another form of this treatment, the "topical" application of the therapeutic composition is targeted at a natural body cavity such as the mouth, pharynx, esophagus, larynx, trachea, pleural cavity, peritoneal cavity, or hollow organ cavities including the bladder, colon or other visceral organs. In this situation, there may or may not be a

significant, primary tumor in the cavity. The treatment targets microscopic disease in the cavity, but incidentally may also affect a primary tumor mass if it has not been previously removed or a pre-neoplastic lesion which may be present within this cavity. Again, a variety of methods may be employed to affect the "topical" application into these visceral organs or cavity surfaces. For example, the oral cavity in the pharynx may be affected by simply oral swishing and gargling with solutions. However, topical treatment within the larynx and trachea may require endoscopic visualization and topical delivery of the therapeutic composition. Visceral organs such as the bladder or colonic mucosa may require indwelling catheters with infusion or again direct visualization with a cystoscope or other endoscopic instrument. Cavities such as the pleural and peritoneal cavities may be accessed by indwelling catheters or surgical approaches which provide access to those areas.

B. Monitoring Gene Expression Following Administration

Another aspect of the present invention involves the monitoring of gene expression following administration of the therapeutic composition. Because destruction of microscopic tumor cells cannot be observed, it is important to determine whether the target site has been effectively contacted with the expression construct. This may be accomplished by identifying cells in which the expression construct is actively producing the gene product. It is important, however, to be able to distinguish between the exogenous gene product and that present in tumor and non-tumor cells in the treatment area. Tagging of the exogenous protein with a tracer element would provide definitive evidence for expression of that molecule and not an endogenous version thereof.

One such tracer is provided by the FLAG biosystem (Hopp *et al.*, 1988). The FLAG polypeptide is an octapeptide (AspTyrLysAspAspAspAspLys) and its small size does not disrupt the expression of the delivered gene therapy protein. The coexpression of FLAG and the protein of interest is traced through the use of antibodies raised against FLAG protein.

Other immunologic marker systems, such as the 6XHis system (Qiagen) also may be employed. For that matter, any linear epitope could be used to generate a fusion protein so long as (i) the immunologic integrity of the epitope is not compromised by the fusion and (ii) the functional integrity is not compromised by the fusion.

X. Therapeutic Formulations and Routes of Administration

Where clinical applications are contemplated, it will be necessary to prepare the viral expression vectors of the present invention as pharmaceutical compositions, *i.e.*, in a form appropriate for *in vivo* applications. Generally, this will entail preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals.

One will generally desire to employ appropriate salts and buffers to render delivery vectors stable and allow for uptake by target cells. Aqueous compositions of the present invention comprise an effective amount of the vector, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrase "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well know in the art. Except insofar as any conventional media or agent is incompatible with the vectors of the present invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

The active compositions of the present invention include classic pharmaceutical preparations. Administration of these compositions according to the present invention will be *via* any common route so long as the target tissue is available *via* that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration may

be by orthotopic, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Such compositions would normally be administered as pharmaceutically acceptable compositions, described *supra*.

The active compounds may be administered *via* any suitable route, including parenterally or by injection. Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial an antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

For oral administration the polypeptides of the present invention may be incorporated with excipients and used in the form of non-ingestible mouthwashes and dentifrices. A mouthwash may be prepared incorporating the active ingredient in the required amount in an appropriate solvent, such as a sodium borate solution (Dobell's Solution). Alternatively, the active ingredient may be incorporated into an antiseptic wash containing sodium borate, glycerin and potassium bicarbonate. The active ingredient may also be dispersed in dentifrices, including: gels, pastes, powders and slurries. The active ingredient may be added in a therapeutically effective amount to a paste dentifrice that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants.

The compositions of the present invention may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups also can be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like. For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration.

"Unit dose" is defined as a discrete amount of a therapeutic composition dispersed in a suitable carrier. For example, in accordance with the present methods, viral doses include a particular number of virus particles or plaque forming units (pfu). For embodiments involving adenovirus, particular unit doses include 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12}, 10^{13} or 10^{14} pfu. Particle doses may be somewhat higher (10 to 100-fold) due to the presence of infection defective particles.

In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, a unit dose could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

In a preferred embodiment, the present invention is directed at the treatment of human malignancies. A variety of different routes of administration are contemplated. For example, a classic and typical therapy will involve direct, intratumoral injection of a

discrete tumor mass. The injections may be single or multiple; where multiple, injections are made at about 1 cm spacings across the accessible surface of the tumor. Alternatively, targeting the tumor vasculature by direct, local or regional intra-arterial injection are contemplated. The lymphatic systems, including regional lymph nodes, present another likely target given the potential for metastasis along this route. Further, systemic injection may be preferred when specifically targeting secondary (*i.e.*, metastatic) tumors.

In another embodiment, the viral gene therapy may precede or following resection of the tumor. Where prior, the gene therapy may, in fact, permit tumor resection where not possible before. Alternatively, a particularly advantageous embodiment involves the prior resection of a tumor (with or without prior viral gene therapy), followed by treatment of the resected tumor bed. This subsequent treatment is effective at eliminating microscopic residual disease which, if left untreated, could result in regrowth of the tumor. This may be accomplished, quite simply, by bathing the tumor bed with a viral preparation containing a unit dose of viral vector. Another preferred method for achieving the subsequent treatment is *via* catheterization of the resected tumor bed, thereby permitting continuous perfusion of the bed with virus over extended post-operative periods.

XI. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1: CONSTRUCTION OF MULTIGENE ADENOVIRAL CONSTRUCTS

Several different gene vectors will be constructed according to the present invention. A first expression cassette will be constructed in a plasmid vector before subcloning in to pIN147 (FIG. 2). The plasmid pIN147 contains bases 1 to 456 and 3333 to 5788 of Ad5 and the CMV IE promoter. The first cassette contains a first gene, followed by the BGH polyA signal, which in turn is followed by an SV40 promoter, a second gene and optionally the SV40 polyadenylation signal, in that order. FIG. 1A shows the resulting cassette with CMV IE promoter from the vector.

A second expression cassette will utilize only one promoter, the CMV IE promoter, derived from pIN147. In this cassette, an IRES will be included downstream of a first gene and a synthetic intron (IVS) and upstream of the second gene and a polyA. FIG. 1B shows the cassette including the upstream pIN147 promoter. The IRES allows for efficient translation of the second gene, and the IVS improves the stability of the mRNA. The IRES and IVS are obtained from plasmids available from Clontech, Palo Alto, CA.

After insertion of the cassettes into the multipurpose cloning site of pIN147, the recombinant vector is contransfected with either pBGH10 or pBGH11, available from Microbix Biosystems, Inc. (Toronto, Ontario, Canada). These plasmids contain bases 1 to 187 and 1340 to 35935 of Ad5, less deletions in the E3 region (28133 to 30818 and 27865 to 30995 and respectively). Recombination will result in the generation of an adenoviral vector containing the multigene cassettes in the E1 region and having an E3-deletion.

Additional constructs will utilize different approaches. Using cassettes including all of the elements shown in FIG. 1A and FIG. 1B, constructs will be generated by inserting the cassettes into pΔE1sp1A and pΔE1sp1B (Microbix Biosystems, Inc.) (FIG.

3), which contain bases 22 to 342 and 3523 to 5790 of Ad5, flanking a multipurpose cloning site. Cotransfection with pBGH10 or pBGH11 permits recombination and generation of adenoviral vectors having E1-inserted cassettes and E3 deletions.

In another construction scheme to generate an adenoviral vector containing two expression cassettes, an expression cassette will be cloned into the adenoviral E3 region of the plasmid pAB26 (Microbix Biosystems, Inc.) (FIG. 4) and *Srfl*-digested adenoviral DNA containing an expression cassette cloned into the E1 region (termed Ad5-expression cassette #1) will be co-transfected into 293 cells and viral particles will be generated as the result of *in vivo* recombination.

To begin this construction scheme, the Ad5-expression cassette #1 vector will be constructed by the co-transfection of pIN147, containing the expression cassette #1 cloned into the E1 region, and pJM17, which contains the entire adenoviral genome including an insertion in the E1 region, into 293 cells. After plaque purification, viral expansion, and subsequent adenoviral DNA purification, the Ad5-expression cassette #1 DNA will be digested with the restriction enzyme *Srfl*, the restriction fragments will be separated by agarose gel electrophoresis, and the band corresponding to approximately 28 kb (or roughly 78% of the adenoviral genome) will be agarose gel purified. The plasmid pAB26 is comprised of Ad5 sequences corresponding to bases 1 to 353, 3825 to 5787, and 24797 to 35935.

An expression cassette will be cloned into the adenoviral E3 multicloning site present within pAB26, and thus will be termed pAB26 expression cassette #2. The co-transfection of the 28 kb *Srfl*-digested Ad5-expression cassette #1 DNA and the pAB26 expression cassette #2 plasmid into 293 cells and the subsequent *in vivo* recombination will generate an adenoviral vector containing two expression cassettes, one in the E1 and the other in the E3 region of the adenoviral genome.

EXAMPLE 2: *IN VITRO* MONITORING OF GROWTH INHIBITION AND APOPTOSIS

Apoptosis assays

DNA Fragmentation Analysis. Following incubation of the cells with the gene therapy construct, cells are harvested, resuspended in 300 µl of PBS with the addition of 3 ml of extraction buffer (10 mM Tris, pH 8.0, 0.1M EDTA, 20 µg/ml RNAse, 0.5% SDS) and incubated at 37°C for 1-2 h. At the end of incubation, proteinase K is added to a final concentration of 100 µg/ml and the solution placed in a 50°C water bath for at least 3 h. DNA is extracted once with equal volumes of 0.5 M Tris (pH 8.0) saturated phenol and then the extraction is repeated with phenol/chloroform. Precipitated DNA is analyzed in a 1% agarose gel.

Cell Fixation. For TUNEL method, the cells are fixed in 1% formaldehyde in PBS (pH 7.4) for 30 min on ice. Cells are then washed with 3 ml of PBS, resuspended in 70% ice-cold ethanol and stored at -20^{0}C until used. For cell-cycle analysis, cells are fixed in 70% ice-cold ethanol only.

Terminal Deoxynucleotidyl Transferase Assay. The assay is performed according to the Gorczyca *et al.*, procedure (Gorczyca *et al.*, 1993). Briefly, after fixation and washing, cells are resuspended in 50 µl of TdT buffer containing 0.2 M sodium cacodylate (pH 7.0), 2.5 mM Tris-HCl, 2.5 mM C_0Cl_2 (Sigma Chemical Company, St. Louis, MO), 0.1 mM DTT (Sigma Chemical Company), 0.25 mg/ml BSA (Sigma Chemical Company), 5 units of terminal transferase (Boehringer Mannheim Biochemicals, Indianapolis, IN), and 0.5 nmoles biotin-16-dUTP along with dATP, dGTP and dCTP at a concentration of 20 µM. Controls are prepared by incubating a separate aliquot of each test sample without d-UTP. The cells are incubated in the solution at 37^{0}C for 30 min, rinsed in PBS, and resuspended in 100 µl of, FITC, the staining solution containing 4X SSC, 0.1% Triton X-100 and 2.5 µg/ml fluoresceined avidin (Vector Labs. Inc., Burlingame, CA). Tubes are incubated for 30 min in the dark at room temperature. Cells are rinsed in PBS with 0.1% Triton X-100 and resuspended in 0.5 ml PBS containing propidium iodide (5

μg/ml) and 70 μl (1 mg/ml) RNAse. Tubes are incubated in the dark on ice for 30 min prior to flow cytometric analysis.

Flow Cytometry Analysis. Samples are analyzed using an EPICS Profile II flow cytometer (Coulter Corp., Hialeah, FL) with the standard optical configuration. At least 5,000 events are collected for each sample. Positively for TdT end-labeling is determined by subtracting the control histogram from the test histogram using the immuno-4 program of the Elite workstation software (Coulter Corp., Hialeah, FL).

Cell Growth Assays.

Cell growth can be measured by cell counting or tritiated thymidine incorporation assays.

Growth Assay by Cell Counting

For cell growth measurements, cells are generally inoculated at densities of 1×10^4 cells in 12 well plates. Cells were trypsinized and counted using a hemocytometer.

Tritiated Thymidine Incorporation Assay

Growth of cells can be measured by analysis of DNA synthesis. Briefly, a stock solution of 100 μCi/ml of ^{3}H-thymidine (Amersham) is prepared by dilution into high glucose DMEM. ^{3}H-thymidine to a final concentration of 1 μCi/ml is added to each well in 20 μl. The reaction is stopped 6 or 15 h later by removal of supernatant from recipient cells. The cells are harvested by the addition of 100X trypsin/EDTA to each well for five min at room temperature. Cells are collected using a Packard Filtermate cell harvester following manufacturer's protocol and washed in distilled deionized water and methanol. Alternatively, the reaction also can be stopped by removing the supernatant from recipient cells and the cells washed once with PBS + 0.5mM $MgCl_2$/1mM $CaCl_2$ and 30 μl of lysis buffer (0.05% SDS/1mM $MgCl_2$/1mM $CaCl_2$) added. The cells are scraped,

adsorbed onto Whatman filters and non-specific radioactivity removed by washing with TCA. Filters are placed into 5 ml scintillant and counted in a gamma counter. The rate of incorporation of activity into DNA is indicative of the rate of cell growth.

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All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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WHAT IS CLAIMED IS:

1. An expression construct comprising:

(a) at least two different genes selected from the group consisting of a tumor suppressor and a cytokine, a tumor suppressor and an enzyme, a tumor suppressor and an antisense oncogene, a tumor suppressor and a toxin, a cytokine and an toxin, a cytokine and an antisense oncogene, an antisense oncogene, a toxin and an enzyme and a toxin, a tumor suppressor and an inducer of apoptosis, a cytokine and an inducer of apoptosis, an antisense oncogene and an inducer of apoptosis, an enzyme and an inducer of apoptosis, and a toxin and an inducer of apoptosis; and

(b) a first promoter active in eukaryotic cells positioned 5' to said different genes.

2. The construct of claim 1, wherein said construct further comprises an internal ribosome entry site (IRES), wherein said IRES is positioned 3' to the upstream gene and 5' to the downstream gene.

3. The construct of claim 1, wherein said construct further comprises a second promoter, wherein said second promoter is positioned 3' to the upstream gene and 5' to the downstream gene.

4. The construct of claim 1, wherein said construct comprises a tumor suppressor and a cytokine.

5. The construct of claim 1, wherein said construct comprises a tumor suppressor and an enzyme.

6. The construct of claim 1, wherein said construct comprises a tumor suppressor and an antisense oncogene.

7. The construct of claim 1, wherein said construct comprises a tumor suppressor and a toxin.

8. The construct of claim 1, wherein said construct comprises a cytokine and an toxin.

9. The construct of claim 1, wherein said construct comprises a cytokine and an antisense oncogene.

10. The construct of claim 1, wherein said construct comprises an antisense oncogene and a toxin.

11. The construct of claim 1, wherein said construct comprises an enzyme and a toxin.

12. The construct of claim 1, wherein said construct comprises a tumor suppressor and an inducer of apoptosis.

13. The construct of claim 1, wherein said construct comprises a cytokine and an inducer of apoptosis.

14. The construct of claim 1, wherein said construct comprises an antisense oncogene and an inducer of apoptosis.

15. The construct of claim 1, wherein said construct comprises an enzyme and an inducer of apoptosis.

16. The construct of claim 1, wherein said construct comprises a toxin and an inducer of apoptosis.

17. The construct of claim 1, wherein said tumor suppressor is selected from the group consisting of p53, p16, p21, Rb, p15, BRCA1, BRCA2, zac1, p73, MMAC1, ATM, HIC-1, DPC-4, FHIT, NF2, APC, DCC, PTEN, ING1, NOEY1, NOEY2, PML, OVCA1, MADR2, WT1, 53BP2, IRF-1 and C-CAM.

18. The construct of claim 1, wherein said cytokine is selected from the group consisting of IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, TNF, GM-CSF, G-CSF, β-interferon and γ-interferon.

19. The construct of claim 1, wherein said enzyme is selected from the group consisting of cytosine deaminase, adenosine deaminase, hypoxanthine-guanine phosphoribosyltransferase, galactose-1-phosphate uridyltransferase, phenylalanine hydroxylase, glucocerbrosidase, collagenase, sphingomyelinase, α-L-iduronidase, glucose-6-phosphate dehydrogenase, HSV thymidine kinase and human thymidine kinase.

20. The construct of claim 1, wherein said oncogene is selected from the group consisting of *ras, myc, neu, raf, erb, src, fms, jun, trk, ret, hst, gsp, bcl-2* and *abl*.

21. The construct of claim 1, wherein said toxin is selected from the group consisting of ricin A chain, diptheria toxin, pertussis, Pseudomonas, *E. coli* enterotoxin, and cholera toxin.

22. The construct of claim 1, wherein said inducer of apoptosis is selected from the group consisting of Bax, Bak, Bcl-X_s, Bik, Bid, Bad, Harakiri, TRAIL, SARP-2, AdE1b and an ICE-CED3 protease.

23. The construct of claim 2, wherein said first promoter is selected from the group consisting of CMV IE, SV40 IE, RSV, β-actin, human ubiquitin C, tetracycline regulatable and ecdysone regulatable.

24. The construct of claim 2, wherein said second promoter is selected from the group consisting of CMV IE, SV40 IE, RSV, β-actin, human ubiquitin C, tetracycline regulatable and ecdysone regulatable.

25. The construct of claim 2, comprising a polyadenylation signal positioned 3' to the downstream gene.

26. The construct of claim 25, comprising (i) a first polyadenylation signal positioned 3' to the upstream gene and 5' to the downstream gene and (ii) a second polyadenylation signal positioned 3' to the downstream gene.

27. The construct of claim 25, wherein said polyadenylation signal is from BGH, thymidine kinase or SV40.

28. The construct of claim 26, wherein said first polyadenylation signal is from BGH or SV40, and said second polyadenylation signal is from BGH when said first polyadenylation signal is from SV40, and said second polyadenylation signal is from SV40 when said first polyadenylation signal is from BGH.

29. The construct of claim 1, wherein said expression construct is a viral vector.

30. The construct of claim 29, wherein said viral vector is selected from the group consisting of retrovirus, adenovirus, lentivirus, vaccinia virus, herpesvirus and adeno-associated virus.

31. The construct of claim 30, wherein said viral vector is retrovirus.

32. The construct of claim 30, wherein said viral vector is adenovirus.

33. The construct of claim 30, wherein said viral vector is vaccinia virus.

34. The construct of claim 30, wherein said viral vector is adeno-associated virus.

35. The construct of claim 30, wherein said viral vector is herpesvirus.

36. The construct of claim 32, wherein said adenovirus vector is replication deficient.

37. The construct of claim 36, wherein said adenovirus vector lacks at least a portion of the E1 region.

38. The construct of claim 37, wherein said adenovirus lacks at least a portion of the E1B region.

39. The construct of claim 38, wherein said adenovirus lacks the entire E1 region.

40. An expression construct comprising

(a) a cytokine gene and an enzyme gene; and

(b) a first promoter active in eukaryotic cells positioned 5' to said genes,

wherein either (i) said cytokine gene is not an IL-2 gene or (ii) said enzyme is not a herpesvirus thymidine kinase gene.

41. A method for the simultaneous expression of two polypeptides in a cell comprising:

(a) providing an expression construct comprising:

(i) at least two different genes selected from the group consisting of a tumor suppressor and a cytokine, a tumor suppressor and an enzyme, a tumor suppressor and an antisense oncogene, a tumor suppressor and a toxin, a cytokine and an toxin, a cytokine and an antisense oncogene, an antisense oncogene, a toxin and an enzyme and a toxin, a tumor suppressor and an inducer of apoptosis, a cytokine and an inducer of apoptosis, an antisense oncogene and an inducer of apoptosis, an enzyme and an inducer of apoptosis, and a toxin and an inducer of apoptosis; and

(ii) a first promoter active in eukaryotic cells positioned 5' to said different genes;

(b) transferring said expression construct into said cell,

whereby expression of said gene is effected.

42. The method of claim 41, wherein said expression construct is a viral vector and said transferring is achieved by viral infection.

43. The method of claim 41, wherein said expression construct is formulated in a liposome and said transferring is achieved by cellular uptake of said liposome.

44. The method of claim 41, wherein said cell is a tumor cell and said cell killed by expression of said different genes.

45. The method of claim 44, wherein said tumor cell is selected from the group consisting of a prostate cancer cell, a lung cancer cell, a brain cancer cell, a skin cancer cell, a liver cancer cell, a breast cancer cell, a lymphoid cancer cell, a stomach cancer cell, a testicular cancer cell, an ovarian cancer cell, a pancreatic cancer cell, a bone cancer cell, a bone marrow cancer cell, a head and neck cancer cell, a cervical cancer cell, a colon cancer cell, a blood cancer cell, an esophagous cancer cell, an eye cancer cell, a gall bladder cancer cell, a kidney cancer cells, a rectal cancer cell, an adrenal cancer cell and heart cancer cell.

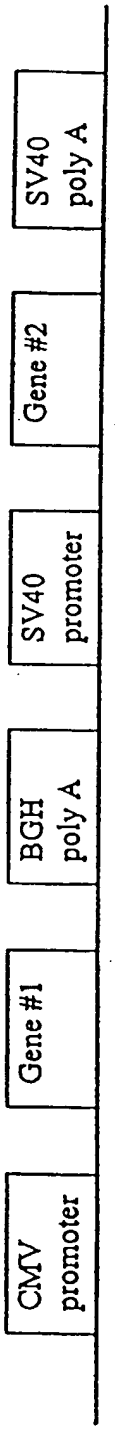
CMV promoter
Gene #1
BGH poly A
SV40 promoter
Gene #2
SV40 poly A

FIG. 1A

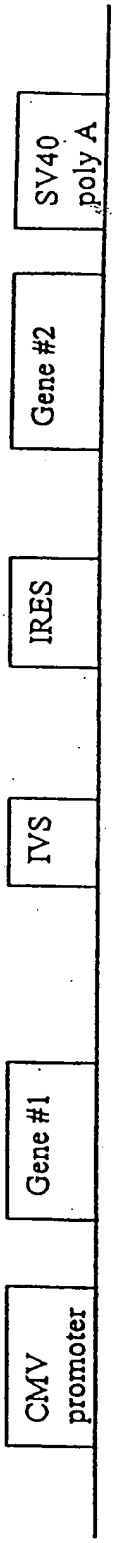
CMV promoter
Gene #1
IVS
IRES
Gene #2
SV40 poly A

FIG. 1B

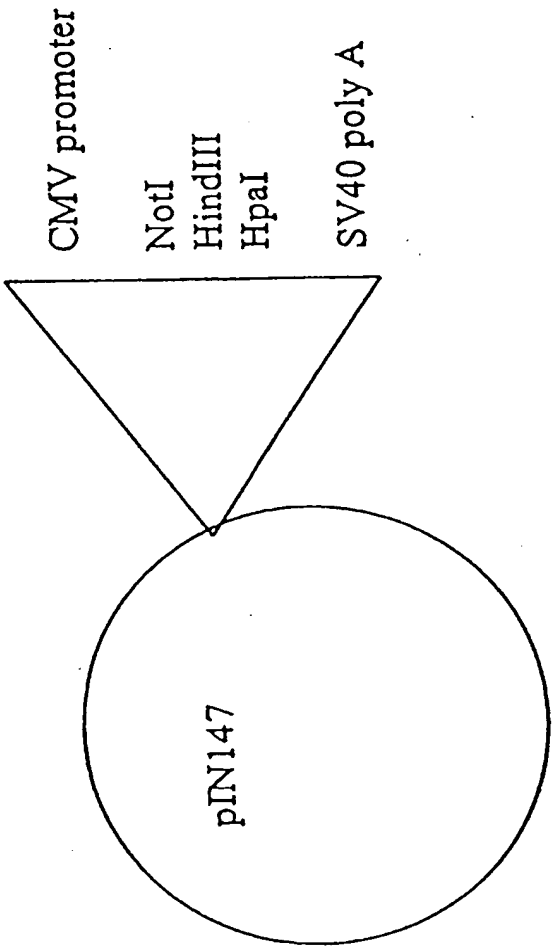

Fig. 2

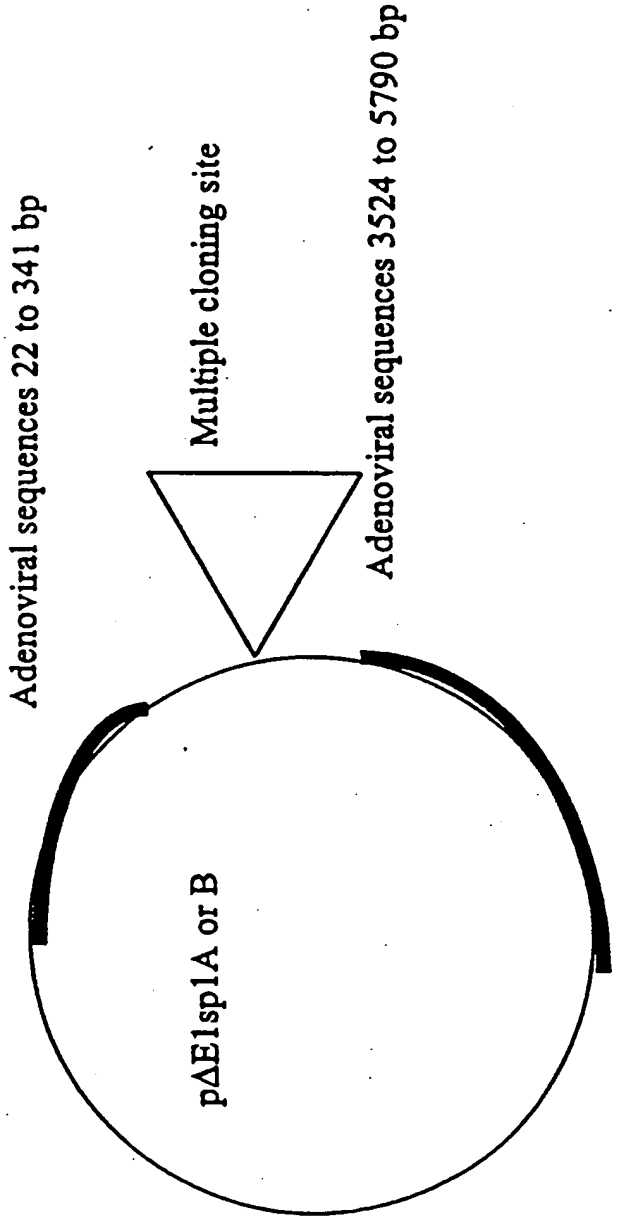
Adenoviral sequences 22 to 341 bp
Multiple cloning site
Adenoviral sequences 3524 to 5790 bp
pΔE1sp1A or B

Fig. 3

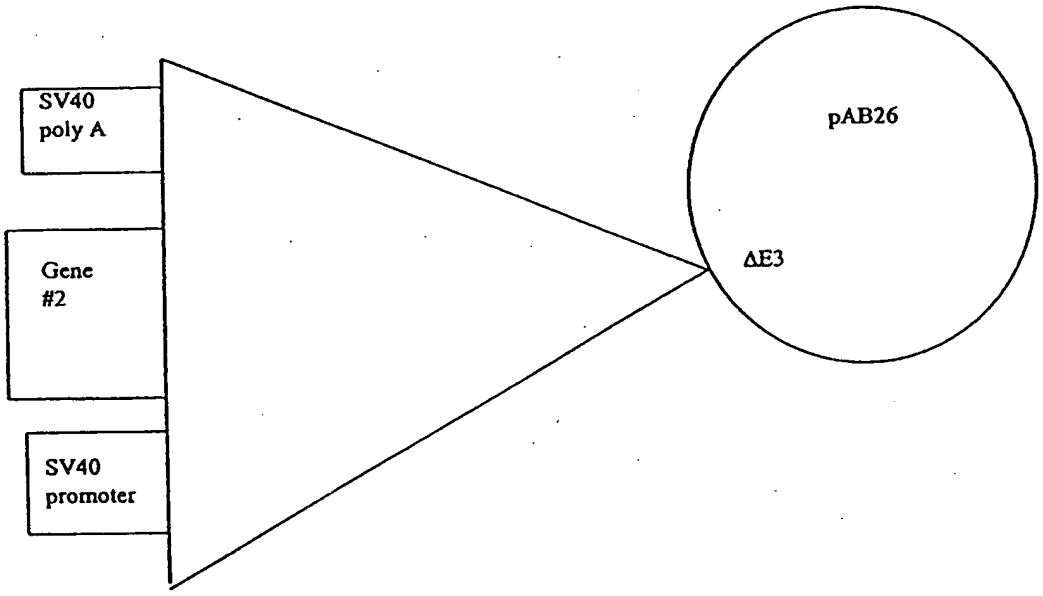

FIG. 4